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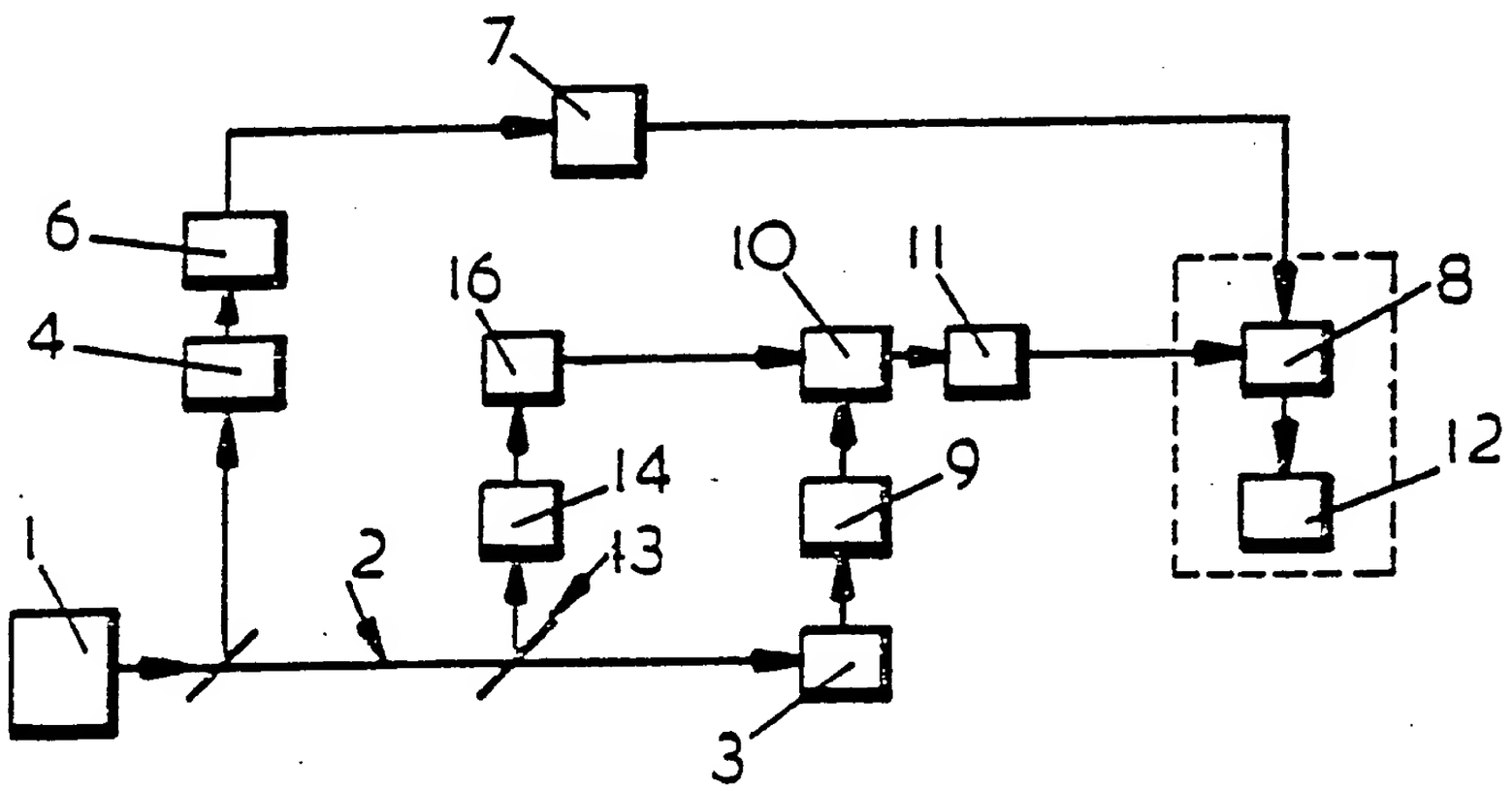

(57) Abstract

Apparatus for measuring fluorescence characteristics of a sample material located at a sample station (3) comprises a fluorescence photon-event receiving means (9), an excitation light source (1) and a means (13, 14, 16) for determining the pulse profile of the excitation light. Detecting means (10) is coupled to receive the output of receiving means (9) and the output of the determining means (13, 14, 16) and synchronisation means (4, 6, 7) is operable by the excitation light source to generate synchronisation pulses for controlling operation of a measuring means (8, 12) which is coupled to the detecting means (10) and which is operable according to the photon correlation technique to provide on a time-shared basis a measure of the fluorescence characteristics and a measurement of the pulse profile.

APPARATUS FOR MEASURING FLUORESCENCE DECAY CHARACTERISTICS OF MATERIALS

This invention relates to apparatus for measuring fluorescence decay characteristics (referred to hereinafter simply as 'fluorescence characteristics') of materials.

Apparatus for measuring fluorescence characteristics of a material is already known and comprises a light source (either narrow band or monochromatic), and hereinafter referred to as an excitation light source, capable of emitting a train of light pulses which are directed onto the material (or sample) under test to excite that sample into a fluorescent state. When the sample fluoresces it emits energy in the form of single photons and the known apparatus comprises photon detection and measuring systems, the latter functioning according to the well-known photon-correlation technique using synchronisation pulses derived from the excitation light source, whereby fluorescence decays and decay times for the sample (and associated properties such as anisotropy decay) are evaluated.

In practise typical excitation light sources are flash-lamps (with associated waveband filters) and lasers both of which suffer from long-term time-dependent changes in their optical pulse profile and it is therefore necessary to measure the excitation pulse profile applied to each sample. In the known apparatus this is undertaken either immediately before or immediately after irradiation of the sample by substituting at the sample station a reflective device or scatterer so that the reflected excitation is directed into the detection and measuring systems. In consequence certain long-term time-dependent changes in the character-istics of the detection and measuring systems are accounted for in addition to long-term excitation pulse profile changes.

The known apparatus suffers from a number of dis-advantages and it is an object of the present invention to obviate or mitigate these disadvantages. For example, the known apparatus measures the excitation pulse profile

before or after sample irradiation and therefore relies upon constancy of excitation pulse profile both during sample irradiation and in continuity with measurement of the excitation pulse profile. In fact known excitation light sources have pulse profiles which tend to vary from pulse to pulse in addition to long-term pulse profile variation. Furthermore, substitution of the sample and reflective device at the sample station requires inter-changeability which in fact is not universally practical because certain samples fluoresce under environmental conditions which render the reflective device difficult to substitute. Additionally, fluorescence usually occurs at a spectral wavelength different from (being larger than) that of the excitation and the detection and measuring systems usually have differing characteristics at these two wavelengths so that the assessment of changes in the detection and measuring systems by directing the excitation light through these systems is inherently inaccurate.

According to the present invention there is provided apparatus for measuring fluorescence characteristics of a material sample, said apparatus comprising

a sample station for receiving a material sample the fluorescence characteristics of which are to be measured,

a fluorescence photon-event receiving means coupled to said sample station to receive single photon events arising therefrom,

an excitation light source capable of emitting a train of excitation light pulses towards said sample station so as to irradiate a sample therein,

excitation pulse profile determining means sensitive to said train of excitation light pulses and having sufficient attenuation to provide an output event count rate compatible with the fluorescence photon event count rate,

detecting means coupled to receive the output of said receiving means and the output of said determining means,

synchronisation means operable by said excitation light source to generate a train of synchronisation pulses,

measuring means coupled to the output of said detecting means and to the output of said synchronisation means and operable according to the photon correlation technique to provide on a time-shared basis a measure of said fluorescence characteristics and a measure of said excitation pulse profile, said measuirng means including discrimination means enabling said excitation pulse profile measure to be distinguished from said fluorescence characteristics measure.

By virtue of the present invention measurement of the excitation pulse profile is effected on a time-shared basis throughout the duration of irradiation of the sample as a result of which the measure is more precise than hitherto and is undertaken without interruption and alteration of the sample station and associated detector so that stringent sample environments can be maintained without disturbance.

The detecting means may comprise a single detector receiving both sets of input events or it may comprise a pair of detectors each receiving only one set of input events. The latter arrangement is preferred because it enables matching of detector transfer functions notwithstanding the different spectral wavelengths of the two sets of input events. Accordingly correction of timing differences is effected automatically.

A time-delay means is preferably provided in one of said receiving means and said determining means, which may be effected optically or electronically, and preferably is arranged to introduce a time-delay of less than one half the pulse repetition period of the excitation light source but substantially greater than the fluorescence decay time

of the sample.

The measuring means is arranged to measure the time interval at which an event occurs in relation to a synchronisation pulse and may convert these measures to proportional amplitude measures. Additionally the measuring means measures the accumulated number of events occurring at each time interval over the duration of the measurement process.

The discrimination means may be provided by electronic routing circuitry whereby fluorescence events are directed into different storage channels from excitation pulse profile events or all events may be stored in a single storage channel and discriminated by virtue of excitation pulse profile events occurring and being stored at longer time interval indicia than fluorescence events.

Embodiments of the present invention will now be described by way of example with reference to the accompanying schematic drawings, in which:

Fig. 1 illustrates the known apparatus for measuring fluorescence characteristics of a sample;

Fig. 2 illustrates a first embodiment of apparatus according to the present invention;

Fig. 3 illustrates a second embodiment of apparatus according to the present invention;

Fig. 4 illustrates a third embodiment of apparatus according to the present invention; and

Fig. 5 illustrates a fourth embodiment of apparatus according to the present invention.

With reference now to Fig. 1 an excitation light source 1 is arranged to emit a train of excitation light pulses along path 2 in order to irradiate a sample in a sample station 3. Fluorescence photon events are received by means 9 which usually is in the form of a wavelength selector such as a grating monochromator the output from which is delivered to a single photon

detector 10 (such as a photomultiplier). Detector 10 issues corresponding output signals to one input of a measuring means 8,12 via a threshold discriminator 11 to eliminate spurious signals. Measuring means 8,12 has a second input which is connected to receive a train of synchronisation pulses derived from source 1, and comprises a device 8 which operates as a start/stop counter and time interval to amplitude converter according to the photon correlation technique and a multichannel analyser 12 in which the output from device 8 is stored as a histogram representing the relative probabilities of occurrance of the various start/stop delay times which is a measure of the fluorescence characteristics of the sample. The synchronisation pulses in Fig. 1 are derived from the optical output of source 1 by way of a photomultiplier 4, discriminator 6 and delay line 7 whereby the synchronisation pulses are arranged to occur after respective detected photon events and accordingly photon events are applied to the 'start' input of device 8 whilst synchronisation pulses are applied to the 'stop' input of device 8.

As is known the synchronisation pulses may be derived electrically from the pulse-forming control system of the source 1 and may be operable upon the 'start' input of the device 8. Additionally, of course, the photon correlation technique yields valid results only when a low detection probability is maintained, typically one photon event for each 100 excitation pulses.

Turning now to the embodiments of the present invention as illustrated in Figs. 2-5 it is to be noted that the components of Fig. 1 are contained therein and retain their respective numerical designations. Thus in Fig. 2 excitation pulse profile determining means is provided in addition to the Fig. 1 components and comprises a beamsplitter 13 located in path 2 whereby a small fraction of the excitation light is delivered to an optical

time-delay means 14 (for example an optical path in air (folded between mirrors) or possibly a light guide such as a single mode glass fibre) the output of which is directed by matching optics 16 to the input of detector 10. Accordingly in this embodiment a single detector 10 receives events on a time-shared basis from element 16 and also from element 9. Discrimination between the two measures provided by means 8,12 is achieved in this embodiment by the position within the memory of analyser 12, excitation events being delayed with respect to fluorescence events.

In the Fig. 3 embodiment the excitation pulse profile determining means incorporates its own detector 23 associated with input optics 22 whereby the geometry of photocathode illumination can be matched with that of detector 10. The output of detector 23 is passed to a time-delay means 25 (in this instance operating electronically) via a threshold discriminator 24 functioning correspondingly to discriminator 11 and the output of means 25 is delivered to the input of means 8,12 in an OR configuration. The Fig. 3 embodiment has the advantage over the Fig. 2 embodiment that the separation of the detection function to two separate detectors 10,23 enables the impulse response of these detectors to be closely matched despite the fact that the detectors are subjected to events in different spectral wavelength regions.

The Fig. 4 embodiment is similar to the Fig. 3 embodiment except for the absence of the time-delay means 25 and the introduction of signal-routing circuitry 18 receiving input signals from discriminators 11,24 and in consequence controlling analyser 12 via line 19 to receive and store excitation pulse profile events separately from fluorescence photon events thereby enabling discrimination between these two measures.

Line 21 provides a reset signal to circuitry 18 following storage of each event. Line 20 provides a confirmatory start signal.

The Fig. 5 embodiment is similar to the Fig. 2 embodiment in that only a single detector 10 is utilised and as regards signal-routing circuitry 18 is similar to the Fig. 4 embodiment but in this instance the input signals to circuitry 18 are derived from device 8 on the one hand and discriminator 17 at the output of detector 15 coupled to the output of delay means 14 on the other hand.

It will be understood that circuitry 18, for example, incorporates a latch which is set to logic 1 or logic 0 depending upon whether a fluorescence event or an excitation event is signalled. The condition of this latch determines the nature of the signal on line 19. The latch is reset by the signal on line 21.

As regards the Fig. 4 embodiment which does not incorporate the time-delay means 25 separation of the two kinds of events is undertaken on the statistical basis that because events are relatively rare the simultaneous presence of both fluorescence and excitation events is exceptional. On this basis either one event only may be recorded as a fluorescence event or both events may be rejected (and neither recorded).

It will now be appreciated that in its simplest form the present invention provides quasi simultaneous measurement (i.e. on a time-shared basis) of both fluorescence characteristics and excitation pulse profile thereby enabling automatic correction of the data collected for fluorescence characteristics to account for variations in the excitation source; both long term and short term variations. This has the additional advantage that such measurement of excitation pulse profile is undertaken independently of the sample station so that special

environments thereat can be maintained as required. In a more complicated form the invention enables matching of the photodetectors to provide similar impulse transfer functions to the received events thereby rendering the detector outputs independent of the spectral wavelength differences between the fluorescence events and the excitation pulse profile events.

CLAIMS

1. Apparatus for measuring fluorescence characteristics of a material sample, said apparatus being characterised by the combination of

a sample station (3) for receiving a material sample the fluorescence characteristics of which are to be measured,

a fluorescence photon-event receiving means (9) coupled to said sample station (3) to receive single photon events arising therefrom,

an excitation light source (1) capable of emitting a train of excitation light pulses towards said sample station (3) so as to irradiate a sample therein,

excitation pulse profile determining means (13,14,16) sensitive to said train of excitation light pulses and having sufficient attenuation to provide an output event count rate compatible with the fluorescence photon event count rate,

detecting means (10) coupled to receive the output of said receiving means (9) and the output of said determining means (13,14,16),

synchronisation means (4,6,7) operable by said excitation light source (1) to generate a train of synchronisation pulses,

measuring means (8,12) coupled to the output of said detecting means (10) and to the output of said synchronisation means (4,6,7) and operable according to the photon correlation technique to provide on a time-shared basis a measure of said fluorescence characteristics and a measure of said excitation pulse profile, said measuring means (8,12) including discrimination means (12) enabling said excitation pulse profile measure to be distinguished from said fluorescence characteristics measure.

2. Apparatus as claimed in claim 1, characterised in that the detecting means (10) comprises a single detector receiving both sets of input events.

3. Apparatus as claimed in claim 1, characterised in that the detecting means (10) comprises a pair of detectors (10,23) each receiving only one set of input events.

4. Apparatus as claimed in any preceding claim, characterised in that a time-delay means (14) is provided in one of said receiving means (9) and said determining means (13,14,16) arranged to introduce a time-delay of less than one half the pulse repetition period of the excitation light source (1) but substantially greater than the fluorescence decay time of the sample.

5. Apparatus as claimed in any preceding claim, characterised in that the measuring means (8,12) is arranged to measure the time interval at which an event occurs in relation to a synchronisation pulse.

6. Apparatus as claimed in claim 5, characterised in that the measuring means (8,12) converts said measure of time interval proportionately to amplitude measures.

7. Apparatus as claimed in either claim 5 or claim 6, characterised in that the measuring means (8,12) measures the accumulated number of events occurring at each time interval over the duration of the measurement process.

8. Apparatus as claimed in any preceding claim, characterised in that the discrimination means (12) is provided by electronic routing circuitry whereby fluorescence events are directed into different storage channels from excitation pulse profile events.

9. Apparatus as claimed in any one of claims 1-7, characterised in that the discrimination means (12) is provided by a single storage channel in which excitation pulse profile events are stored at longer time interval indicia than fluorescence events.

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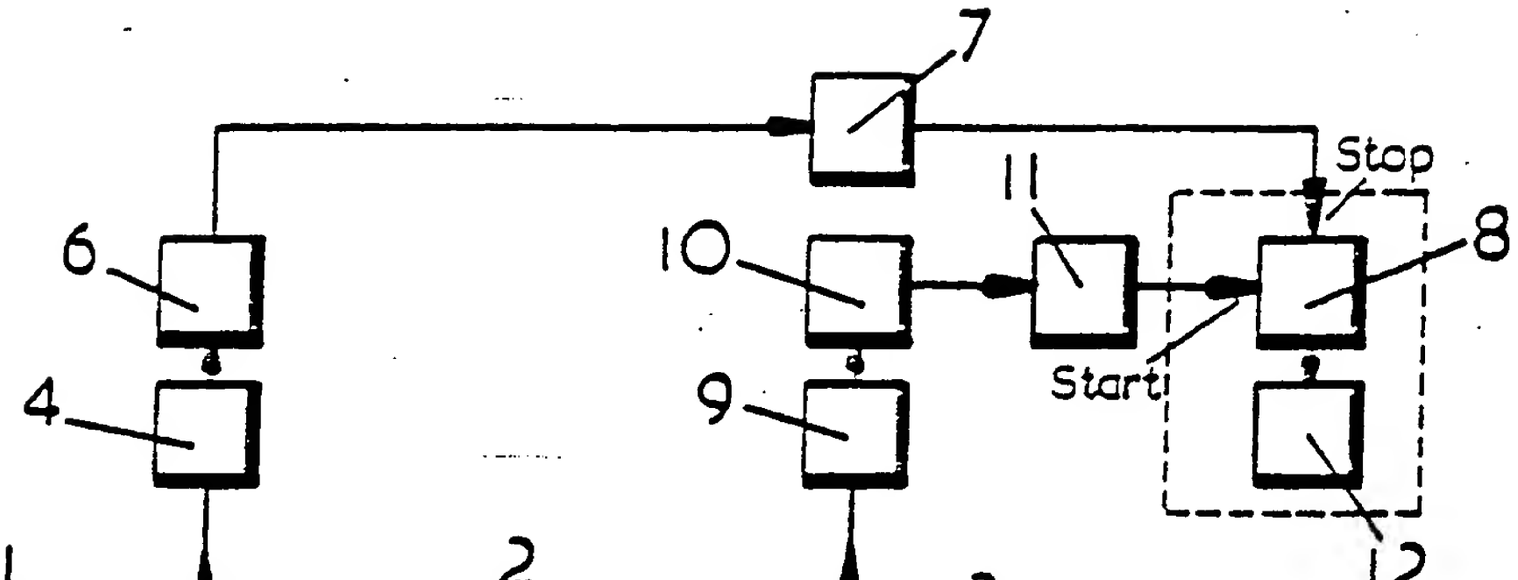

FIG. 1

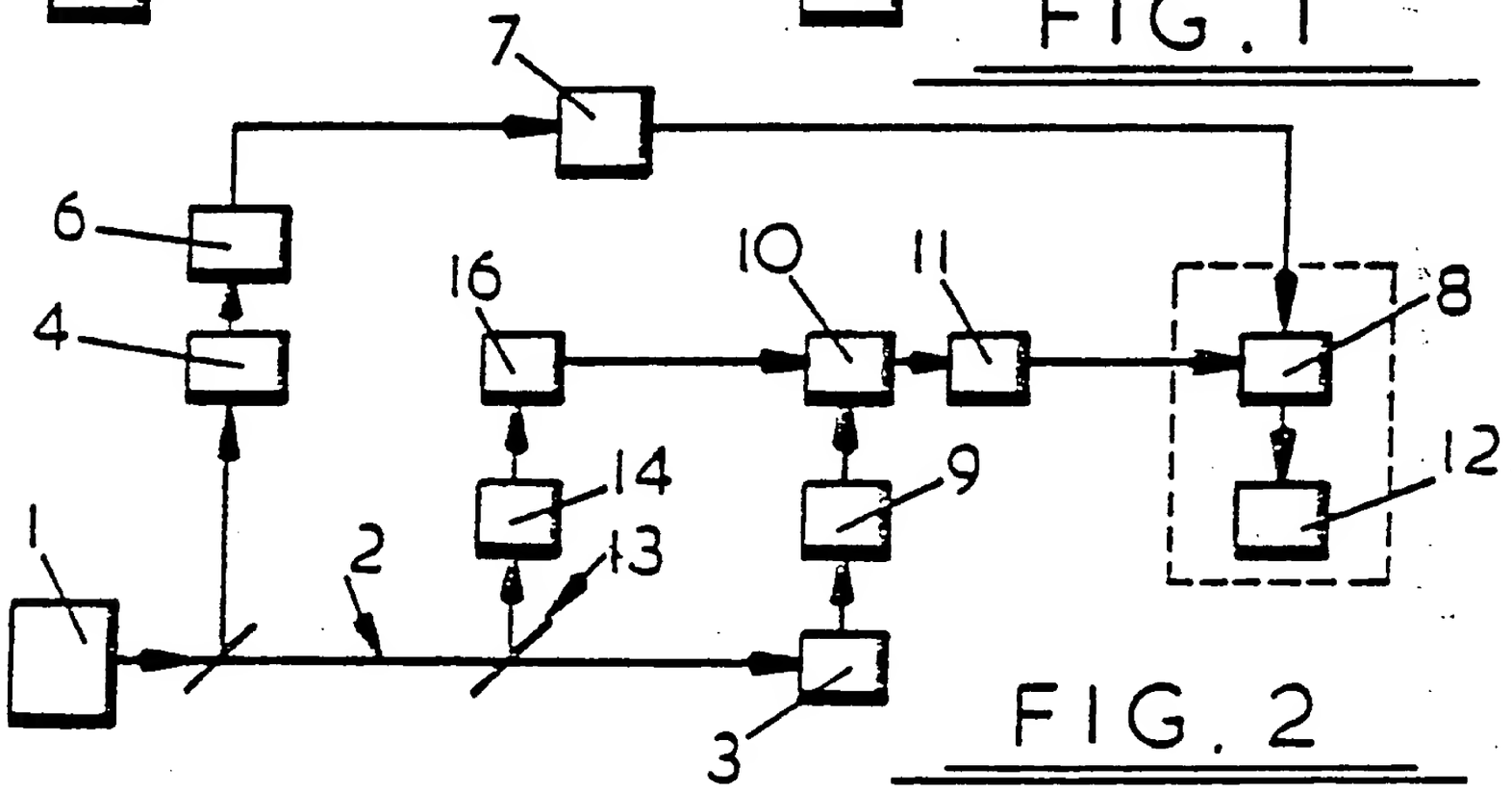

FIG. 2

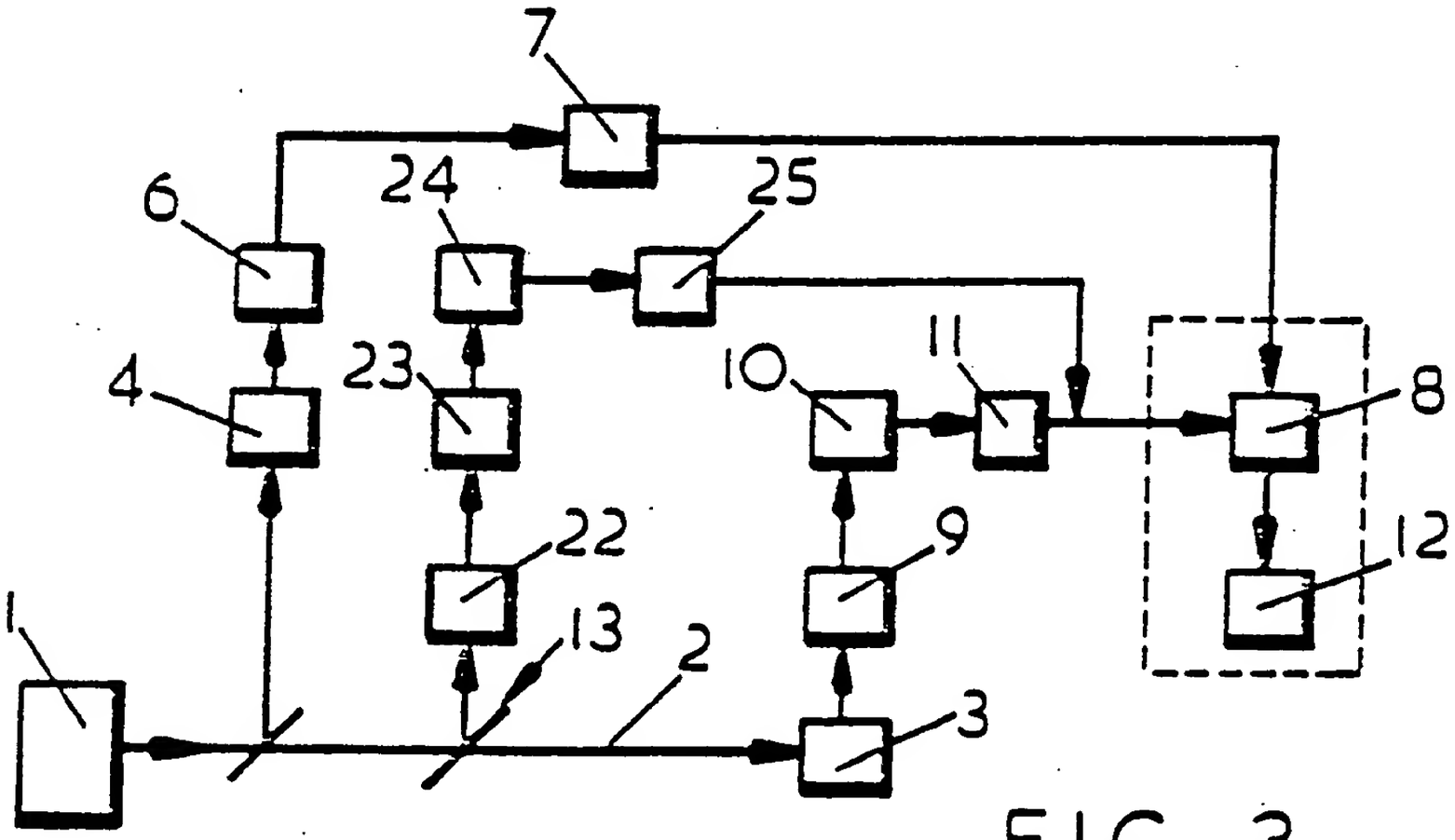

FIG. 3

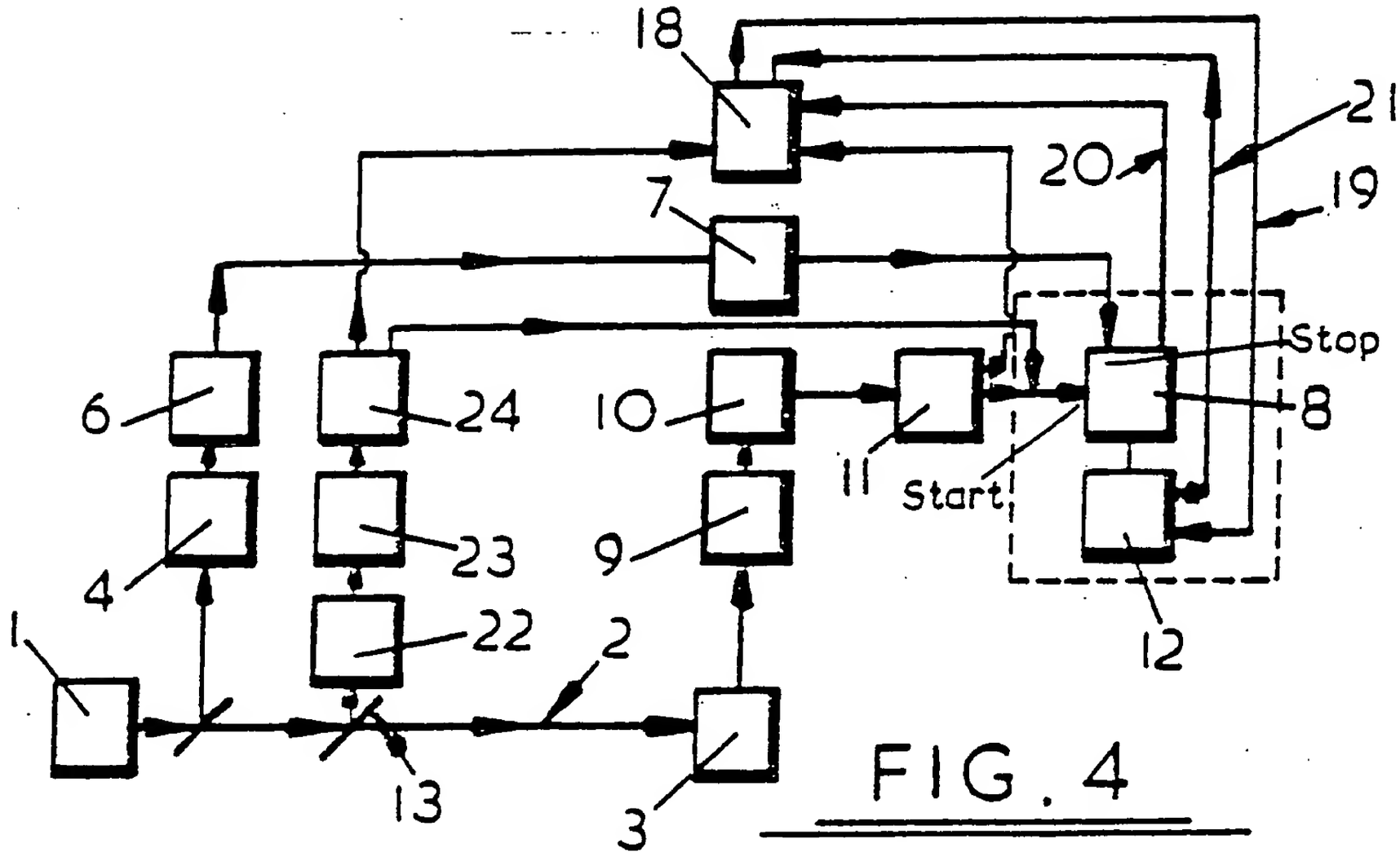

FIG. 4

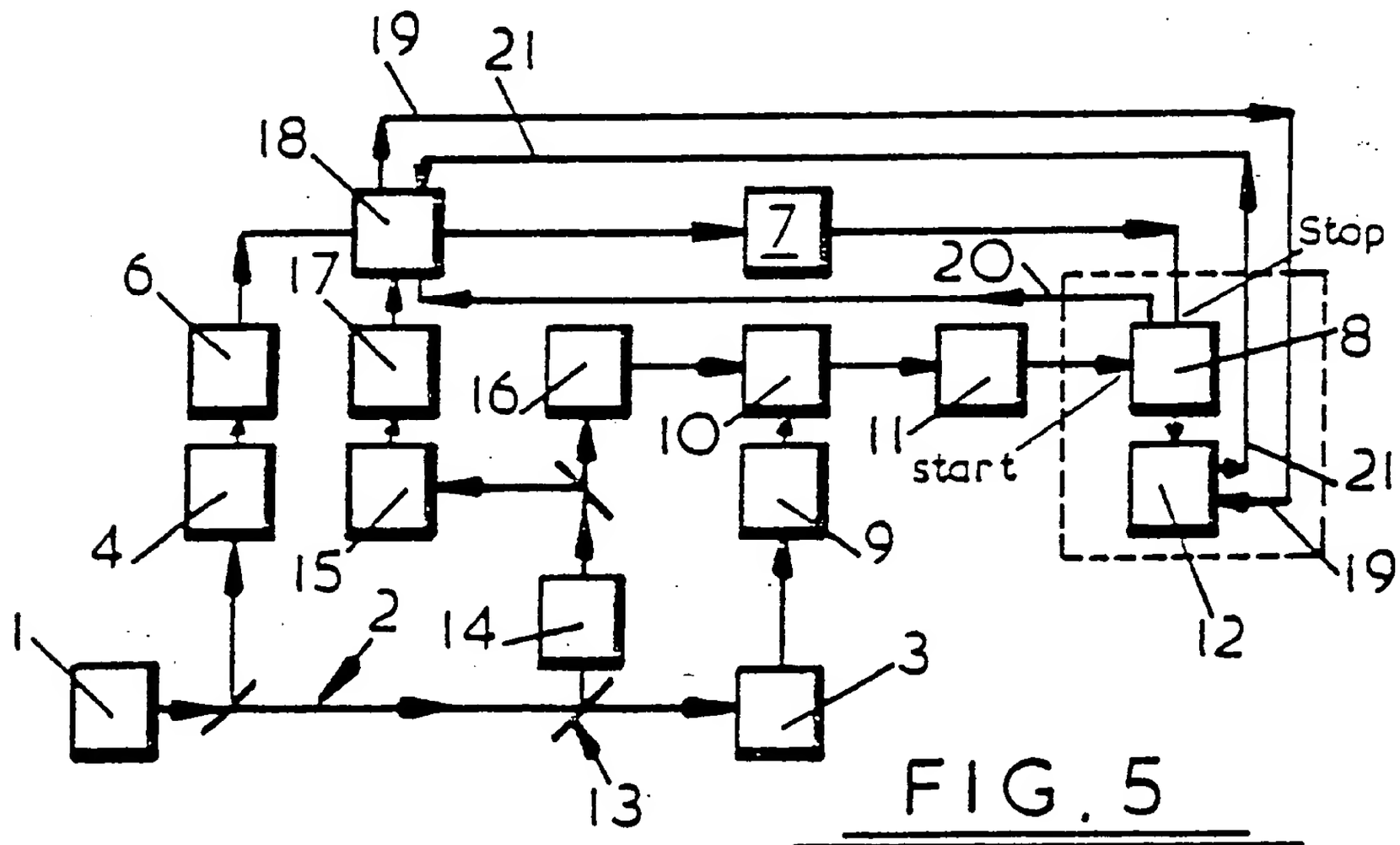

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00028

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 21/64

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC[4]	G 01 N 21/64

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
A	Journal of Physics E: Scientific Instruments, volume 10, no. 10, October 1977, Dorking (GB) D. Birch et al.: "A single photon counting fluorescence decay time spectrometer", pages 1044-1049, see page 1045, paragraph 2 "Description" --	1,5,6
A	Review of Scientific Instruments, volume 48, no. 8, August 1977 D. Rayner et al.: "Correction of instrumental time response variation with wavelength in fluorescence lifetime determinations", pages 1050-1054, see page 1050, lines 24-38; pages 1051-1052 paragraph "Description of correction technique" --	1

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* Special categories of cited documents: 10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd April 1985	31 MAI 1985
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Review of Scientific Instruments, volume 53, no. 9, September 1982 R. Wijnandts van Resandt et al.: "Double beam fluorescence lifetime spectrometer with subnano-second resolution", pages 1392-1397, see page 1392, lines 34-60; page 1393, lines 1-30; page 1394, lines 15-26	1
P,X	Journal of Physics E: Scientific Instruments, volume 17, no. 5, May 1984, Dorking (GB) D. Birch et al.: "Differential pulse fluorometry using photo-multipliers", pages 417,418, see page 417, paragraph "Introduction and paragraph 2 "Summary"	1,3-6,8

XP 000635855

An all solid-state near-infrared time-correlated single photon counting instrument for dynamic lifetime measurements in DNA sequencing applications

G01N21/64F

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We have constructed a simple, all solid-state, time-correlated single photon counting device for collecting decay profiles of chromophores attached to DNA fragments moving through a capillary tube filled with a sieving gel under the influence of an applied electric field (capillary electrophoresis). The major components of the instrument consist of an actively pulsed GaAlAs diode laser ($\lambda_{excitation}$=780 nm; τ_p<200 ps; repetition rate=80 MHz; average power=5.0 mW), single photon avalanche diode (dark count rate <50 cps; quantum efficiency=65% at 800 nm) and a PC board containing a constant fraction discriminator, time-to-amplitude converter, and an analog-to-digital converter (maximum processing count rate=3×10^6 cps). The instrument possessed a response function of approximately 275 ps (full width at half-maximum), adequate for measuring fluorescence lifetimes in the subnanosecond regime. To demonstrate the utility and the sensitivity of the instrument, dynamic measurements of fluorescence lifetimes for near-IR dye-labeled DNA fragments were measured during capillary electrophoresis for the identification of the dye-labeled nucleotide bases via temporal discrimination. The results indicated that in a two-dye experiment, in which only two of the four constituent bases which comprise DNA were labeled with unique fluorescent probes, the characteristic lifetime of the probe could be used to readily identify the terminal nucleotide base. Decay profiles were constructed from roughly 17 500 photoelectrons accumulated over a 2 s counting interval at a loading level of approximately 6.2×10^{-21} mol (3900 molecules) of DNA per electrophoretic band. The lifetimes of the two labeling dyes were determined to be 669 ps (±42 ps) and 528 ps (±68 ps). © *1996 American Institute of Physics.*
[S0034-6748(96)04911-8]

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I. INTRODUCTION

Tremendous efforts are currently being invested into developing new technologies and instrumentation for sequencing the human genome, which consists of 3×10^9 nucleotide bases, due to the time-consuming and labor-intensive steps required to obtain sequence data using existing state-of-the-art instruments. For obtaining DNA sequences, the common approach is to use the Sanger dideoxy chain termination method,[1] in which a series of DNA fragments are randomly terminated at one of the constituent nucleotide bases, which comprise the biopolymer. The next step involves a fractionation process, in which the DNAs are size separated using electrophoretic methods, with the terminal nucleotide base identified (base calling) during the separation from which the original sequence is constructed. The major thrusts of the technology development are directed toward increasing the speed of acquiring sequencing data, construction of multiplexing instruments to improve throughput, and improving the accuracy in base calling during the separation process.

The approach typically employed for identifying the terminal base involves laser-induced fluorescence, due to its ease of use, high sensitivity, and the ability to easily automate the detection. The four constituent bases (adenine, A, cytosine, C, guanine, G, and thymine, T) are attached to one of four fluorescent probes, which possess unique emission maxima, allowing spectral discrimination for identification.[2-5] While this has been a fairly robust method and is the technique commonly incorporated into many commercial DNA sequencers, the protocol does present itself with potential difficulties, including the need for multiple excitation sources and/or detection channels and cross talk between detection channels due to spectral overlap which can produce errors in identifying the nucleotide bases during the sequence analysis. In high throughput instruments, where many sequencing lanes must be run in parallel, the necessity for multiple detection channels to process the fluorescence from each label can make the sequencing device instrumentally intensive.

We have recently suggested an approach to base calling in DNA sequencing applications using fluorescence lifetime discrimination of dyes with similar absorption and emission maxima, but possess unique fluorescence lifetimes.[6] Several advantages are associated with lifetime discrimination for base calling, including:

(1) the calculated lifetime is immune to concentration differences;
(2) the fluorescence lifetime can be determined with higher precision than fluorescence intensities; and
(3) only one excitation source is required to efficiently excite the fluorescent probes and only one detection chan-

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nel is needed to process the fluorescence for appropriately selected dyes.

One of the potential difficulties arising from the use of lifetime discrimination in base-calling applications for DNA sequencing is the poor photon statistics (limited number of photocounts) produced from the need to make a dynamic measurement (the chromophore is resident in the excitation beam for only 1–5 s) and the low mass loading levels associated with electrophoresis, especially in capillary electrophoresis. For example, it is not uncommon to encounter a mass loading range of 0.001–1.0 amol of DNA per electrophoretic band.

Recently, we demonstrated that time-resolved near-IR (NIR) fluorescence,[7] in conjunction with a simple computational method, known as the maximum likelihood estimator (MLE),[8] could be used to determine the lifetimes of C-terminated DNA fragments separated via capillary gel electrophoresis with high accuracy and precision. NIR fluorescence has some decisive advantages compared to the more traditional visible fluorescence methods in that matrix interferences, in the form of Raman and Rayleigh scattered light from the gel matrix and fluorescence impurities are significantly reduced compared to visible excitation, significantly improving the accuracy and precision in the lifetime measurement.[7] In those experiments, a passively mode-locked Ti:sapphire laser pumped by the all-lines output of an Ar ion laser and conventional time-correlated single photon counting electronics were used to analyze the time-resolved fluorescence of DNA fragments labeled with a NIR dye.

Another potential difficulty associated with lifetime measurements in DNA sequencing applications is the need for sophisticated and complex instrumentation. For the time-domain techniques, the instrumentation basically consists of a mode-locked, pulsed-laser (Nd:YAG), fast photodetector (microchannel plate photomultiplier) and extensive electronics (constant fraction discriminator, time-to-amplitude converter, analog-to-digital converter, and pulse-height analyzer, typically NIM bin modules). As such, the time-correlated single photon counting (TCSPC) instrument can make the measurement difficult, especially for those not well trained in laser operations.

An additional advantage of implementing NIR fluorescence, is that solid-state diode lasers and photodetectors can be used to construct a simple fluorescence detection apparatus. In this article, we wish to discuss the development of a TCSPC apparatus which uses all solid-state components (pulsed diode laser and avalanche photodiode) and basically is turn-key in operation. While others have shown that pulsed diode lasers can be used for TCSPC applications, these devices did not possess the ability to make dynamic measurements of chromophores with subnanosecond lifetimes with sufficient sensitivity to determine lifetimes for small amounts of fluorescing material.[9,10] The excitation source of our instrument consists of a pulsed GaAlAs diode laser with a single photon avalanche diode (SPAD) serving as the photodetector. In addition, the system utilizes a PC board in which all of the electronics needed for making a TCSPC measurement are situated. The use of this instrument

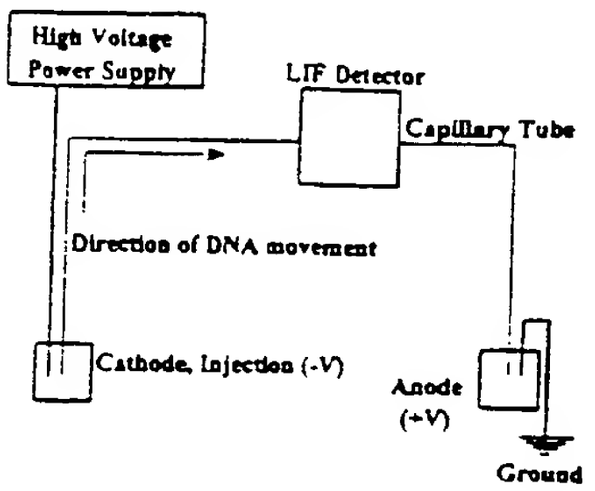


FIG. 1. Block diagram of the NIR TCSPC instrument. L, laser singlet focusing lens; C, capillary; BD, beam dump; MO1, collecting microscope objective; MO2, focusing microscope objective; SPAD, single photon avalanche diode; AMP, amplifier; CFD, constant fraction discriminator; TAC, time-to-amplitude converter; ADC, analog-to-digital converter; and MCS, multichannel scaler. Also shown in this figure is a conventional capillary electrophoresis system for size-fractionation of DNAs. The injection end of the capillary is held at a negative potential, causing all DNAs to migrate toward the anodic end of the capillary and past the detector.

will be demonstrated in DNA sequencing applications by identifying bases in a two-dye labeled experiment with the oligonucleotides separated via capillary gel electrophoresis (CGE).

II. INSTRUMENTATION

NIR-LIF Detection system. A block diagram of the NIR laser-induced fluorescence (LIF) system for capillary electrophoresis utilizing the pulsed diode laser source (BiosQuant GmbH, model DL-4040, Berlin Germany) and TCSPC board (BiosQuant GmbH, model SPC-300, Berlin Germany) is shown in Fig. 1. The laser source was an actively pulsed solid-state GaAlAs diode with a repetition rate of 80 MHz, and an average power of 5.0 mW at a lasing wavelength of 780 nm. The laser beam was converted from an elliptical to circular shape using external cavity correction optics. The diode head was driven by an electrical short pulse generator which supplied high repetition rate picosecond current

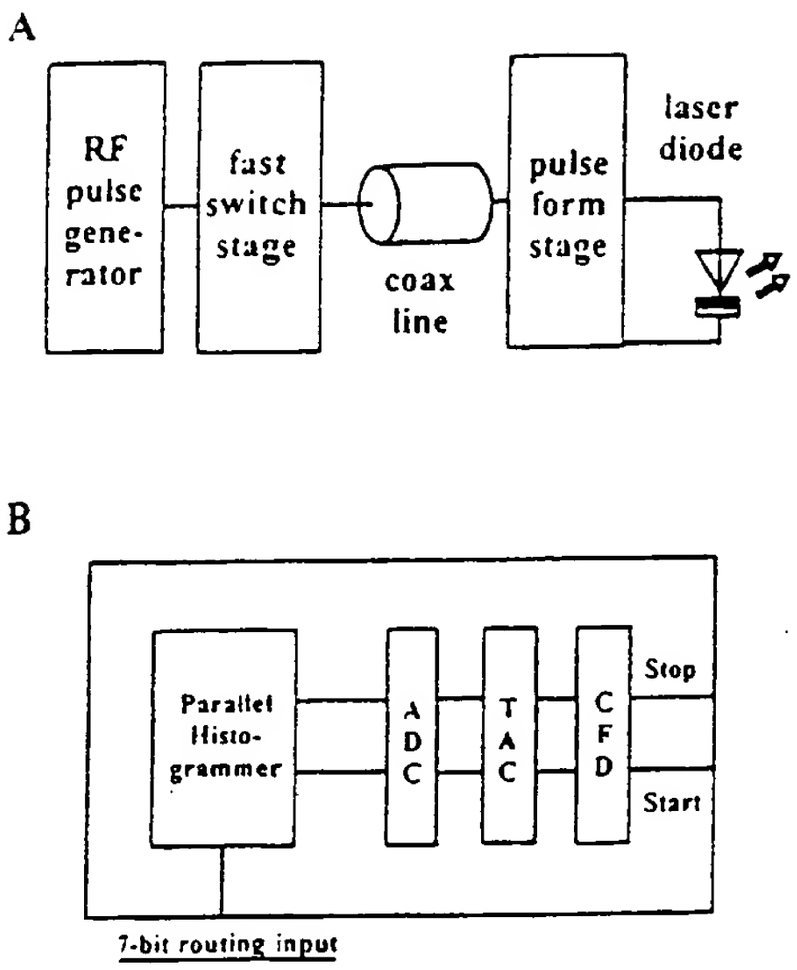


FIG. 2. Block diagram of the electronics for the pulsed diode laser (A) and the TCSPC board (B).

pulses. The driver consisted of a rf pulse generator, fast switching stage, coax line driver, and a pulse shaper stage [see Fig. 2(A)].[11] The driver delivered 3.6 W (50 Ω) current pulses at 80 MHz to the diode head with a full width at half-maximum (FWHM) of 500 ps.

The laser was focused onto a capillary tube to an approximately 14 μm ($1/e^2$) beam waist using a diode laser singlet lens (Melles Griot, Irvine, CA). The emission was collected in a conventional 90° format with a 40× high numerical aperture (NA) microscope objective (Nikon, Natick, MA, NA=0.85) and the emission spatially filtered with a slit (width=0.4 mm). The fluorescence was further isolated from scattering photons using an eight cavity interference bandpass filter (center wavelength=850 nm, half bandwidth=30 nm, Omega Optical, Brattleborough, VT). The filtered fluorescence was then focused onto the photodetector by a 10× microscope objective producing an image of approximately 20 μm on the face of the photodetector.

The photodetector was a single photon avalanche diode (SPAD, EG&G Electrooptics Canada, Vaudreuil, Canada) stationed on a thermoelectric cooler with a photoactive area of 1.77×10^{-4} cm^2 (i.d.=150 μm) and a dark count rate of approximately 100 counts/s. The pulses from the photodetector were amplified (Phillips Scientific, Mahwah, NJ) 20-fold and sent to the time-correlated single photon counting board. The board, which plugs directly into the PC-bus, consisted of a constant-fraction discriminator, time-to-amplitude converter, analog-to-digital converter, and a multichannel analyzer with 128 parallel channels [see Fig. 2(B)].[12,13] The electronics have a dead time of <260 ns, allowing efficient processing of single photon events at counting rates exceeding 2 mil counts/s. The timing jitter in the electronics was determined to be <20 ps. The electronics allowed collection of 128 sequential decay profiles with a timing resolution of 9.77 ps per channel. The controlling software was written in LabView (National Instruments, Austin, TX).

In the present experiments, each decay profile was collected for 2 s during the electrophoresis, allowing a total data acquisition time of 256 s after which time, the data were dumped to memory producing a down time of approximately 20 s. The steady-state fluorescence intensity was acquired using a PC-resident time/counter board (Computer Boards, INC, model CIO-CTR05, Mansfield, MA). Counts were accumulated for 1 s intervals of time during the electrophoresis to construct the fluorescence intensity electropherograms.

III. CAPILLARY GEL ELECTROPHORESIS

The capillary gel columns, which consisted of a 5% crosslinked polyacrylamide gel (75 μm i.d., 375 μm o.d., Polymicro, Phoenix, AZ) were prepared using published procedures.[14] The column was cut to a total length of 60 cm, with the distance from injection to laser detection being 53 cm. The voltage to the capillary column was supplied by a Spellman high voltage power supply (CZ1000R, Plainview, NY). In all cases, the electrophoresis was performed using a field strength of 250 V/cm. DNA samples were inserted onto the gel column using an electrokinetic mode, in which the capillary tube was inserted into a DNA sample, a 15 kV voltage applied for 90 s, and then placing the capillary tube back into the running buffer subsequent to performing the electrophoresis. The running buffer for the electrophoresis consisted of a TRIS buffer, with borate and EDTA (pH=8.3) containing 7.3 M urea as the denaturant.

IV. PREPARATION OF DNA SEQUENCING LADDERS

The DNA sequencing ladders were prepared using standard Sanger sequencing methods.[1,15,16] The DNA primers (oligonucleotides which consist of, in the present case, 17 bases and bind to a specific site on the target DNA) were covalently labeled with one of two NIR dyes, IRD40 or IRD41 (Li-COR, Lincoln, NE). The structures of these dyes along with their absorption/emission maxima, fluorescence lifetimes, and electrophoretic mobilities are shown in Fig. 3. IRD40 was used to label DNA fragments terminated in A- and IRD41 was used for labeling of the C-terminated DNA fragments in the sequencing experiments.

V. RESULTS AND DISCUSSION

Due to the similar absorption and emission maxima of the labeling fluorescent dyes used in this experiment (see Fig. 3), spectral discrimination for nucleotide base identification would be difficult. However, the dyes selected show distinct fluorescence lifetimes ($\Delta\tau_f$=69 ps), which should permit facile identification using lifetime discrimination for an instrument which possesses a response function adequate for measuring subnanosecond lifetimes. In order to determine the lifetimes of the dye/DNA primers, a static experiment (nonflowing conditions) was performed using long photon integration times and high dye concentrations along with a nonlinear least squares method to determine τ_f. The results indicated lifetime values of 650 ps for IRD40/DNA and 581 ps for IRD41/DNA, both adequately described by a

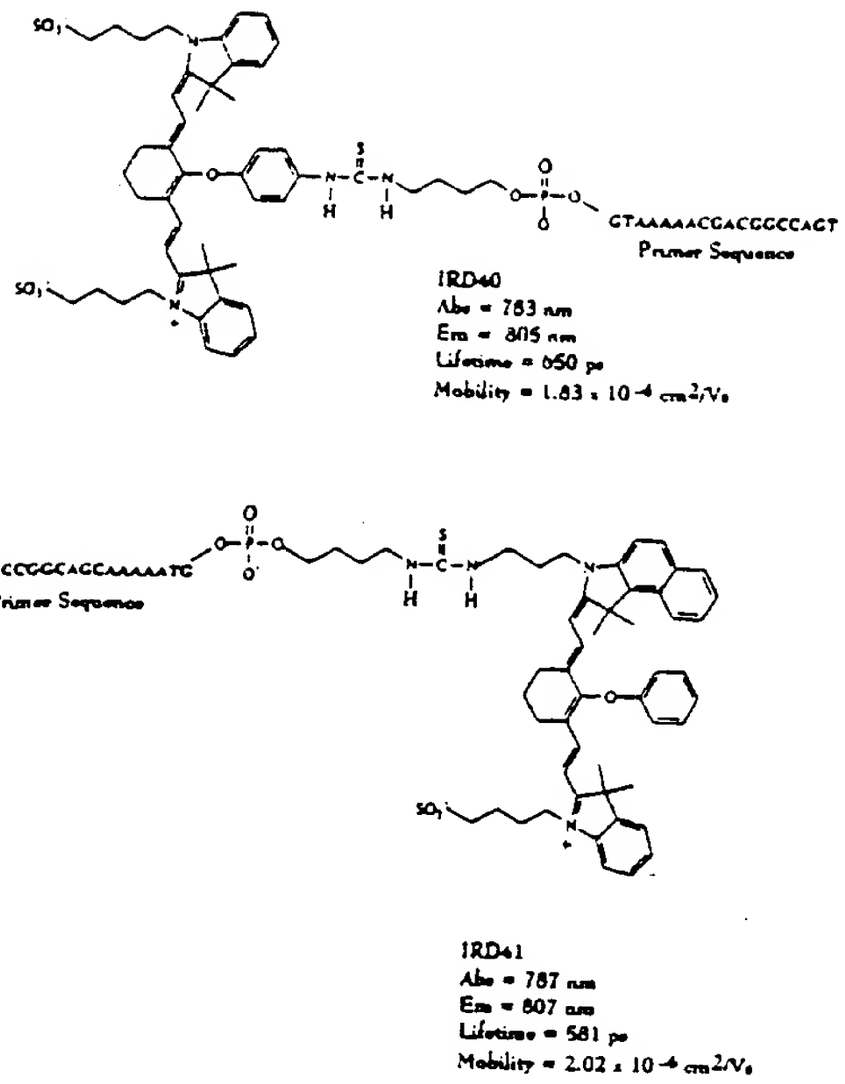


FIG. 3. Chemical structures of the NIR-labeled DNA sequencing primers. Also shown is the electrophoretic mobility of the dye/DNA molecules and their absorption and emission maxima, which were determined in an unpolymerized gel matrix consisting of 5% acrylamide, TRIS/borate buffer with EDTA (pH=8.3) and the urea (denaturant).

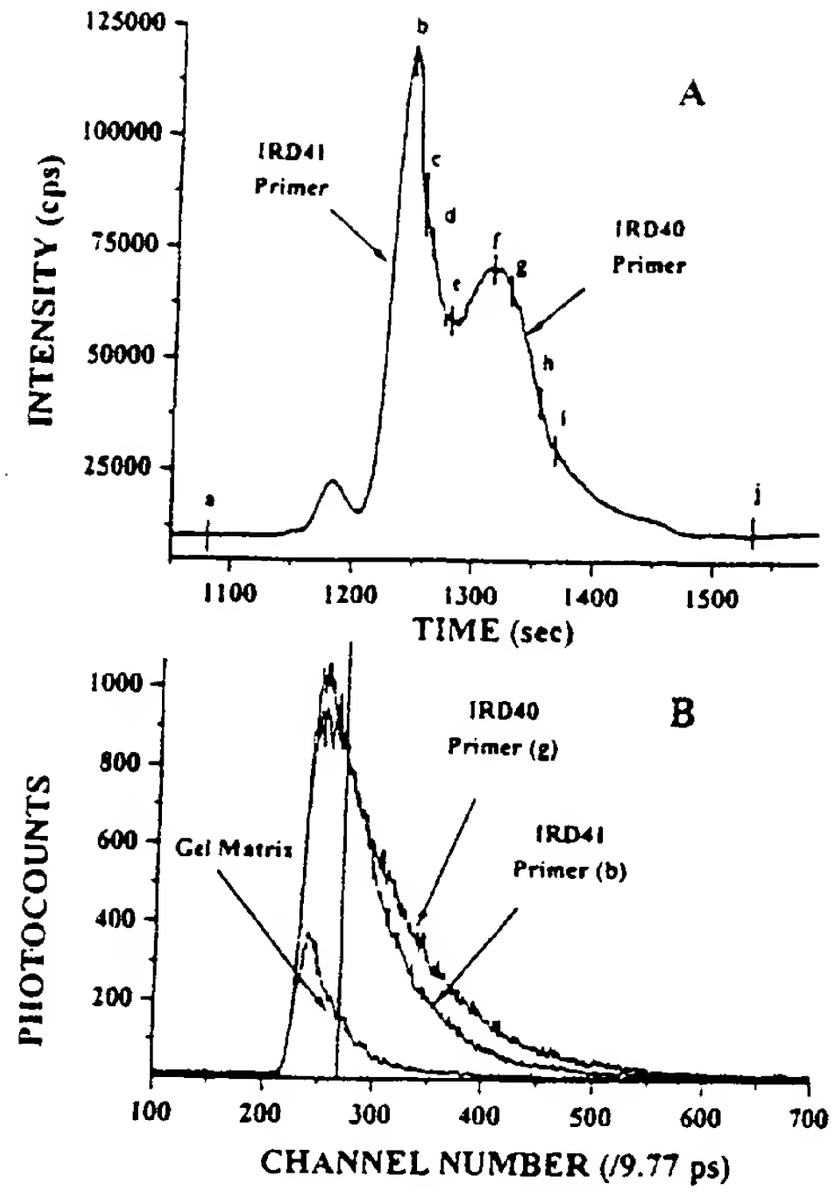


FIG. 4. Steady-state fluorescence intensity vs electrophoresis time (A) and time-resolved decay profiles (B) of the gel matrix, IRD40 and IRD41 dye-labeled DNA primers. The small letters in (A) indicate the time intervals during the electrophoresis over which the decay profiles were collected. The solid line shown in 2(B) indicates the start channel for calculation of the fluorescence lifetime.

monoexponential function ($\chi^2 \sim 1.03$). In this case, the lifetimes of these probes were determined in an unpolymerized gel matrix with urea (DNA sequencing conditions), since the photophysics of these type of dyes have been shown to depend dramatically upon the solvent system.[17]

Figure 4(A) shows the steady-state fluorescence intensity versus electrophoresis time for the dye-labeled DNA primers only electrophoresed in the capillary gel column. In this experiment, each electrophoretic band contained approximately 3.1×10^{-18} mol of dye-labeled DNA, which was calculated from the apparent electrophoretic mobility of the dye/primers in the electric field, injection conditions, and the dye concentration. As can be seen in Fig. 4, two bands are present, even though both dyes are attached to the same sized DNA primer. From charge considerations only (IRD41 is neutral and IRD40 is anionic), one would expect that the IRD40-labeled primer would migrate faster than the IRD41-labeled primer. However, the point of attachment of the dye to the primer linkage also affects the frictional factor which influences the mobility, resulting in a faster migration rate for the IRD41 dye/primer, irrespective of charge considerations only.[14] In Fig. 4(B) is shown the decay profiles for the gel matrix and the fluorescently labeled primers integrated over the points indicated in Fig. 4(A). The instrument response function (IRF), determined from the gel only curve, was found to be 275 ps FWHM. In order to calculate the fluorescence lifetimes for these dye/DNA bands on-line during the electrophoresis, maximum likelihood estimators were used, which is given by the following relation:[18]

$$1+(e^{T/\tau f}-1)^{-1}-m(e^{mT/\tau f}-1)^{-1}=N_t^{-1}\sum_{i=1}^{m} iN_i. \tag{1}$$

where N is the total number of photocounts in the decay profile, T is the time width of each bin (9.77 ps), m is the time interval over which the lifetime was calculated (4.3 ns), i is the time bin number, and N_i is the number of counts in the ith time bin. A table was constructed from the left-hand side of Eq. (1) using different lifetime values and the experimental data were used to evaluate the right-hand side of Eq. (1) in order to determine τ_f. The relative precision in the measurement using this relationship is simply given by $N_t^{-(1/2)}$ when $\tau_f \gg T$. In order to minimize the amount of scattering photons included into the calculation for the lifetimes, the determination was carried out over a time interval that was shifted by 97 ps [see the solid line in Fig. 4(B)] from the channel containing the maximum number of photocounts with 440 channels (4.3 ns) included into the calculation.

The calculated lifetimes for the points indicated in Fig. 4(A) are shown in Table I along with the total number of counts included into the calculation as well as the standard deviation in the measurement, which was determined from $N_t^{-(1/2)}$ multiplied by the observed lifetime. As can be seen from these results, the apparent lifetimes for points a and j, where only the gel matrix contributes to the decay, was

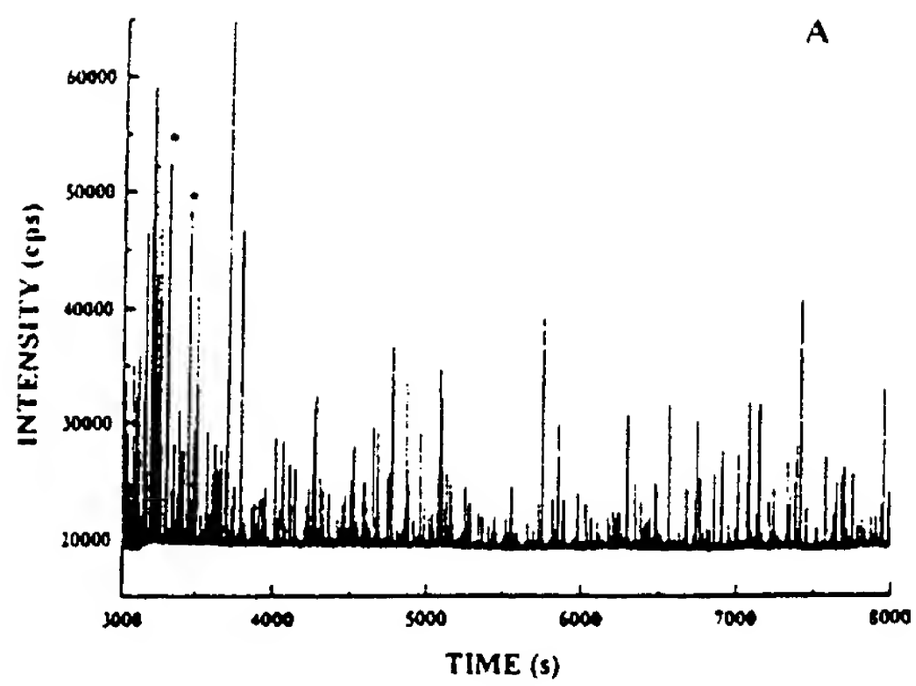
TABLE I. Calculated fluorescence lifetimes for the NIR dye-labeled DNA sequenching primers. Also shown, are the number of photocounts included in the calculation as well as the standard deviation in the measurement. The letters indicate the points during the electropherogram in which the decay profiles were constructed [see Fig. 4(A)]. Each decay profile was integrated over a 2 s time interval.

Time point	Lifetime (ps)	Num. photocounts[a]	Standard deviations[b] (ps)
a	422	11 060	±4
b	585	73 945	±2
c	582	71 336	±2
d	595	68 654	±2
e	645	46 844	±3
f	660	58 784	±3
g	662	53 727	±3
h	666	31 731	±4
i	661	23 270	±4
j	412	12 152	±6

[a]Includes only those counts used in the lifetime calculation.
[b]Calculated by multiplying $N_t^{-(1/2)}$ by the calculated lifetime.

found to be approximately 412 and 422 ps, respectively, but with significantly fewer photocounts included in the calculation when compared to the case where dye-labeled DNA was present. When dye/DNA was resident within the detection volume, the lifetime values varied, with the value determined by the identity of the dye. At point *b*, a value of 585 ps was found, which corresponds to the value calculated in the static experiment for IRD41. At point *d*, the lifetime value is seen to be greater than that expected for IRD41 alone. This results from the fact that the algorithm used to calculate the lifetime cannot differentiate between a mono- and multiexponential decay, and the calculated lifetime represents a weighted average of the various components comprising the decay. At this point during the electrophoresis, both IRD40 and IRD41 are resident within the detection zone. At points *f*–*i*, the lifetime values are similar to that calculated for IRD40 in the static experiment, since only this dye is expected to be present in the detection zone.

In Fig. 5(A) is shown the steady-state fluorescence intensity versus electrophoresis time for A- and C-terminated DNA sequencing fragments separated by capillary gel electrophoresis. In this particular experiment, IRD41 was used to label the DNA fragments that were terminated in a C and IRD40 was used for labeling the A-terminated DNA fragments. Since only two dyes were available, the *T*'s and *G*'s were not fluorescently visible. In Fig. 5(B) is shown the decay profiles for the gel matrix, IRD41 and IRD40 dye-labeled DNA fragments. As can be seen, the number of photocounts in this case is substantially less than that found in the case of the dye/primers only (see Fig. 4). Therefore, the scattering photons make a larger contribution into the decay, which can potentially introduce bias into the calculated lifetime. Over the time interval in which the lifetime was calculated, 17 373 counts were included in the lifetime determination for the IRD41 DNA fragment and 26 096 counts for the IRD40 DNA fragment [see the marked bands in Fig. 5(A)]. Based upon the apparent electrophoretic mobilities of these two fragments, the electrokinetic injection conditions, dye/primer concentration, and the approximate extension length



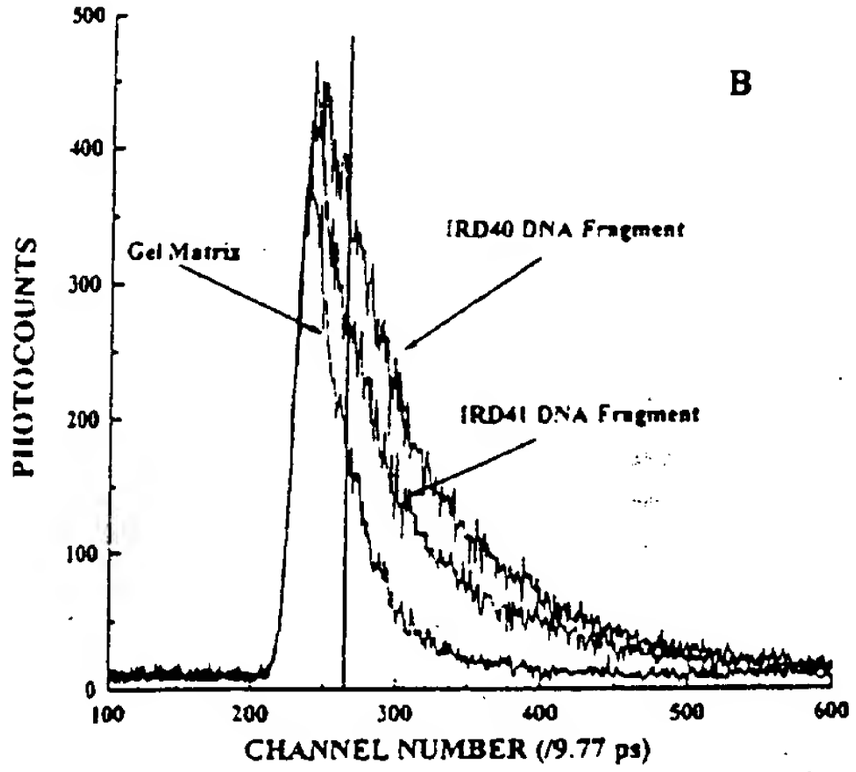


FIG. 5. Steady-state fluorescence intensity electropherogram (A) of A- and C-terminated DNA fragments separated by capillary gel electrophoresis. The A fragments were labeled with IRD40 and the C fragments with IRD41. In (B) is shown the time-resolved decay profiles for the gel matrix and two bands taken from (A), which are indicated with an asterisk.

for DNA polymerization, we estimated that the marked bands in Fig. 5(A) contain approximately 6.2×10^{-21} mol (3700 molecules) of dye-labeled DNA.

In Fig. 6 is shown an expanded view of the intensity electropherogram shown in Fig. 5(A) along with the identity of the terminal base and some of the fluorescence lifetimes calculated using Eq. (1). Over the entire electropherogram, the average lifetime value determined for the IRD40 A-terminated DNA fragments was found to be 669 ps, with a standard deviation of ±42 ps, while for the IRD41 C-terminated DNA fragments, the average lifetime was 528 ps and a standard deviation of ±68 ps. Both of these values are similar to the lifetime values calculated in the static case (within experimental error), however, the standard deviations in these measurements were larger than expected based upon the average number of counts included into the determination. For the A fragments, the average number of photocounts in the lifetime calculation was found to be 19 928 and for the C fragments, 10 781 counts, which would result in standard deviations (determined from $N_t^{-(1/2)}x\tau_f$) of ±5 and ±6 ps, respectively, if the precision in the measurement was

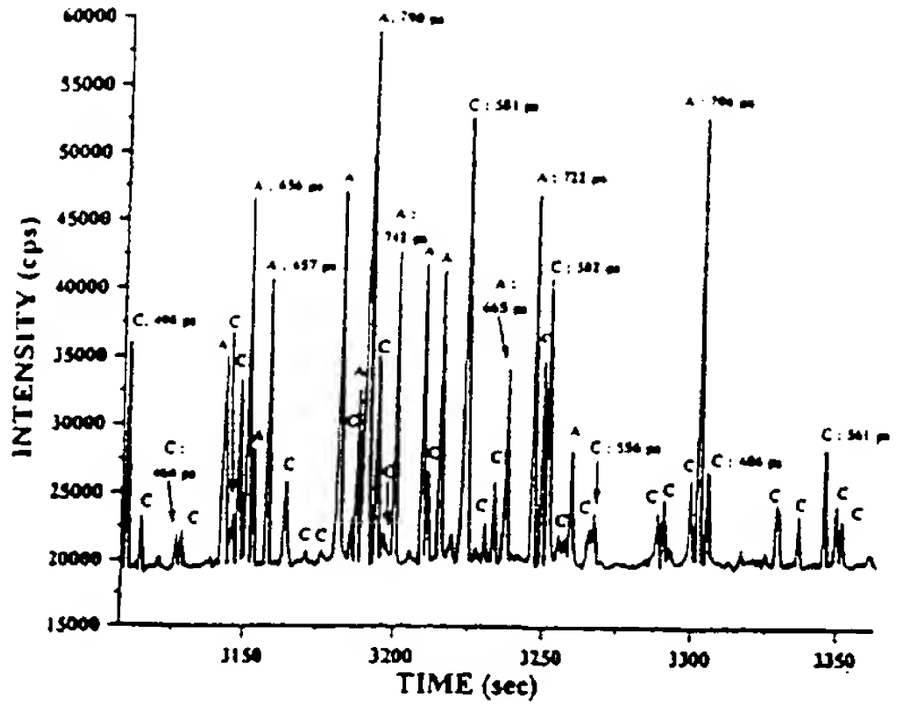


FIG. 6. Expanded view of the intensity electropherogram shown in Fig. 5(A) with the bases identified via lifetime discrimination.

determined primarily by photon statistics. The large standard deviation in these experiments results from the fact that the TCSPC electronics sequentially collects the decay profiles over 2 s intervals during the electrophoresis. In some cases, the data accumulation time for construction of the decay profile may not be aligned (in time) with the time interval over which the electrophoretic band is resident within the laser beam. This would result in a decay profile that was constructed on the rising or falling edge of the electrophoretic band and inclusion of a disproportionately large amount of scattering photons, biasing the determination to lower lifetime values. In addition, if two bands were present in the detection zone during decay profile construction, the calculated lifetime would represented a weighted average of both dyes. These situations could be corrected by including a threshold condition into the hardware and/or software, in which the decay profiles would be collected only over time intervals in which the steady-state counting rate exceeded the average background rate by some predetermined value.

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Flexible instrument for time-correlated single-photon counting

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A single-photon counting instrument is described that is capable of recording single decay curves, time-resolved spectra, and sequences of fluorescence decay curves with the emission wavelength, the time from the start of the experiment, or other externally variable values as parameters. The instrument contains an internal microcomputer for experiment and measurement system control, data processing, and data management. It can be operated at pulse repetition rates ranging from 10 to 200 MHz and allows a photon count rate of up to 10^6/s. The FWHM of the instrument response is 94 ps with a photomultiplier tube of type 18 ELU-FM.

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I. INTRODUCTION

Time-correlated single-photon counting (TCSPC) is widely known to be a powerful method for fluorescence detection.[1] It has the advantage of wide dynamic range, single photon sensitivity, and high time resolution (in the ns and even in the ps time scale). With the advent of mode-locked argon-ion lasers and synchronously pumped dye lasers, replacing the excitation light sources with kHz repetition rate,[2] ultrashort optical pulses at high repetition frequency have become readily available. The instrumental resolution of a TCSPC system and the measurement time to get a fluorescence decay curve with a satisfactory signal-to-noise ratio now depend mainly on the parameters of the photon detector and of the signal processing system. The full width at half maximum (FWHM) value of the instrumental response [instrumental response width (IRW)] is commonly used to describe the time resolution of a system. Because of the highly statistical nature of the signal,[1] the accuracy with which time constants of measured light events can be extracted from TCSPC measurement data by means of deconvolution analysis is about 10 times better than the IRW resolution value. From this point of view, comparing TCSPC systems with other time-resolved measurement techniques like optical sampling oscilloscope, synchroscan streak camera, and all other types of streak cameras,[3] a TCSPC system with IRW of 80 ps can be regarded as having the same effective time resolution as, e.g., a synchroscan streak camera of 15 ps, because the improvement in effective time resolution (as discussed above) is much less dramatic (an improvement factor of about 2).

Using a photomultiplier (PMT) focused by static crossed electrical and magnetic fields, an IRW of 47 ps has been produced.[4] Recently, using a microchannel-plate PMT, the IRW could be decreased down to 30 ps.[5] Furthermore, using single-photon avalanche photodiodes, an IRW of 28 ps at room temperature was achieved.[6,7] Until now less attention was paid to the maximum counting rate, which mainly determines the time to measure a fluorescence decay curve.

In this paper a microcomputer-controlled instrument for time-correlated single-photon counting is described that contains an internal microcomputer to control the measurement system modules, data processing, and management. All modules of the measuring system are connected to the common microcomputer bus and controlled by the instrument software. This principle allows a great variety of modes of operation and yields a high flexibility of the system. The instrument software allows recording of decay curves, of time-resolved spectra, and of sequences of decay curves depending on the wavelength, the time from the start of the experiment, or any other externally variable parameter. For direct inspection of light pulse shapes, a SPC oscilloscope mode is provided. With a suitable optical system a multiplexed recording on four channels and a digital lock-in technique is available in all operating modes.

II. ELECTRONIC SYSTEM

A. Measuring system

The electronic system of the instrument is shown in Fig. 1. The synchronization signal (SYN) derived from the laser pulse sequence is fed to the frequency divider (FRD). The division ratio of the FRD is software selectable from 2:1 to 8:1. It determines the number of the signal periods recorded.

The single-photon pulses (SPP) from the photon detector are fed to the constant fraction trigger (CFT). The CFT delivers an output pulse at the crossover point of the input pulse and the delayed input pulse. Since the temporal position of the crossover point is independent of the pulse amplitude, this timing method minimizes the time jitter due to the amplitude jitter of the detector pulse. The time walk of the CFT is <50 ps in an amplitude range of 1:2 and <100 ps in an amplitude range of 1:10.

The single-photon pulses are fed simultaneously to the window discriminator DIS1. DIS1 suppresses the processing of detector pulses outside the optimal amplitude range of the CFT and of small background pulses from the detector. The CFT output pulses are used to start the time-amplitude converter (TAC).

Compared to systems operating with flash lamps, the start and stop inputs of the TAC are inverted.[8] This enables the processing of the high pulse repetition rate of the cw laser (typically 125 MHz) since the TAC has to oper-

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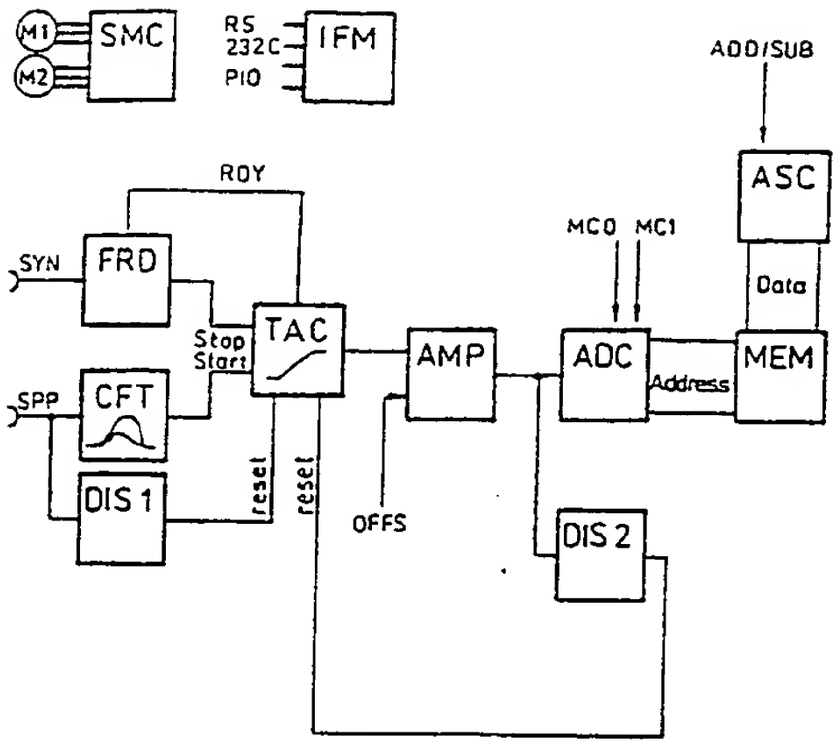

FIG. 1. Measuring system (FRD = frequency divider, CFT = constant fraction trigger, DIS1, DIS2 = window discriminators, TAC = time-to-amplitude converter, AMP = amplifier, ADC = analog–digital converter, MEM = memory, ASC = addition/subtraction circuit, IFM = interface module, SMC = step motor control module).

ate only at the considerably lower photon count rate (<1 MHz). The inversion of the time axis resulting from the inverted start/stop operation is not crucial, since the true time axis can be restored by reversed readout of the data from the memory.

The TAC output voltage is fed to the amplifier (AMP). The gain factor of the amplifier can be chosen by the microcomputer to vary the time scale. The measured time can be shifted by means of the microcomputer-controlled offset signal (OFFS).

The subsequent analog–digital converter (ADC) converts the amplified TAC signal. The resolution of the ADC can be set by the microcomputer to 8, 9, or 10 bits, resulting in a length of the recorded decay curves of 256, 512, or 1024 points. The memory address consists of the ADC output word and two additional memory control bits MC0 and MC1. By these bits the single photon events are routed to four different memory parts, providing a multiplexed quad channel operation of the instrument.

The content of the addressed memory location is incremented or decremented by a computer-selectable value via the addition/subtraction circuit (ASC). Incrementing or decrementing is controlled by the external signal ADD/SUB. This allows the combination of the time-correlated single-photon counting with a digital lock-in technique.[9,10]

The maximum count rate of the instrument is important for reducing the real time taken by the measurement. The processing rate is limited mainly by the speed of the ADC and of the memory. In order to increase this speed, the ADC and MEM modules have been so designed that an A/D conversion can be started already during the operation of storing a preceding measurement in the memory.

A further considerable improvement of the count rate is obtained by introducing the window discriminator DIS2. This discriminator inhibits the conversion of TAC pulses outside the time window of interest, which is often considerably shorter than the time range covered by the amplified TAC pulses. Maximum count rates from 10^5 to $10^6\ s^{-1}$ are attained, depending on the selected ADC resolution.

Unless the probability of having an event (photon or dark current pulse) within the analyzed time range after the excitation is very small (less than 1%), the shape of the optical signals measured by time-correlated photon counting is distorted.[11] Essentially, the effect arises since the TAC records only the first occurring event in all cases where more than one falls within the measured time range. This is often referred to as "pulse pile-up effect." It is worth noting, however, that with the high-repetition rate (125 MHz) laser pulses the rate of data accumulation is very high even with event probability much smaller than 1%, that is, with negligible pile-up effect. Furthermore, the effect can be accurately evaluated and corrected, taking into account the number of excitation pulses employed; in fact, a suitable correction routine[11] can be readily included in the software for the treatment of data.

For the control of the external optical setup the instrument contains the step motor driver module SMC and the interface module IFM with two RS 232 C channels. The measuring system modules have been especially developed in our laboratory. In contrast to commonly used systems, they are fully software programmable. All modules are connected directly to the microcomputer system.bus. The main parameters of the modules are adjustable via the microcomputer. Control and data processing functions are implemented in the software as far as possible. This results in a reduced hardware expenditure in the measuring system and in a high flexibility of the instrument.

B. Software

Because of the close connection between the instrumentation system and data processing, the software is an inseparable component of the instrument. The software package consists of several modules which are loaded by the program manager in dependence on the selected function.

The measurement routines contain the programming of the measuring system modules, the timing of the experiment, and the monochromator stepping required by the selected operation mode. The measurement can be controlled by the operator via a command interpreter function. The command interpreter includes adjusting the FRD ratio, TAC range, AMP gain and offset, DIS2 discrimination levels, ASC count increment, collection time intervals, stop conditions, and monochromator wavelength.

III. MODES OF OPERATION

A. Measurement of light pulse shapes

This mode is applied to the recording of decay curves at selected wavelengths. The measurement can be terminated either after the programmed collection time or upon the first overflow in the measurement data memory. In

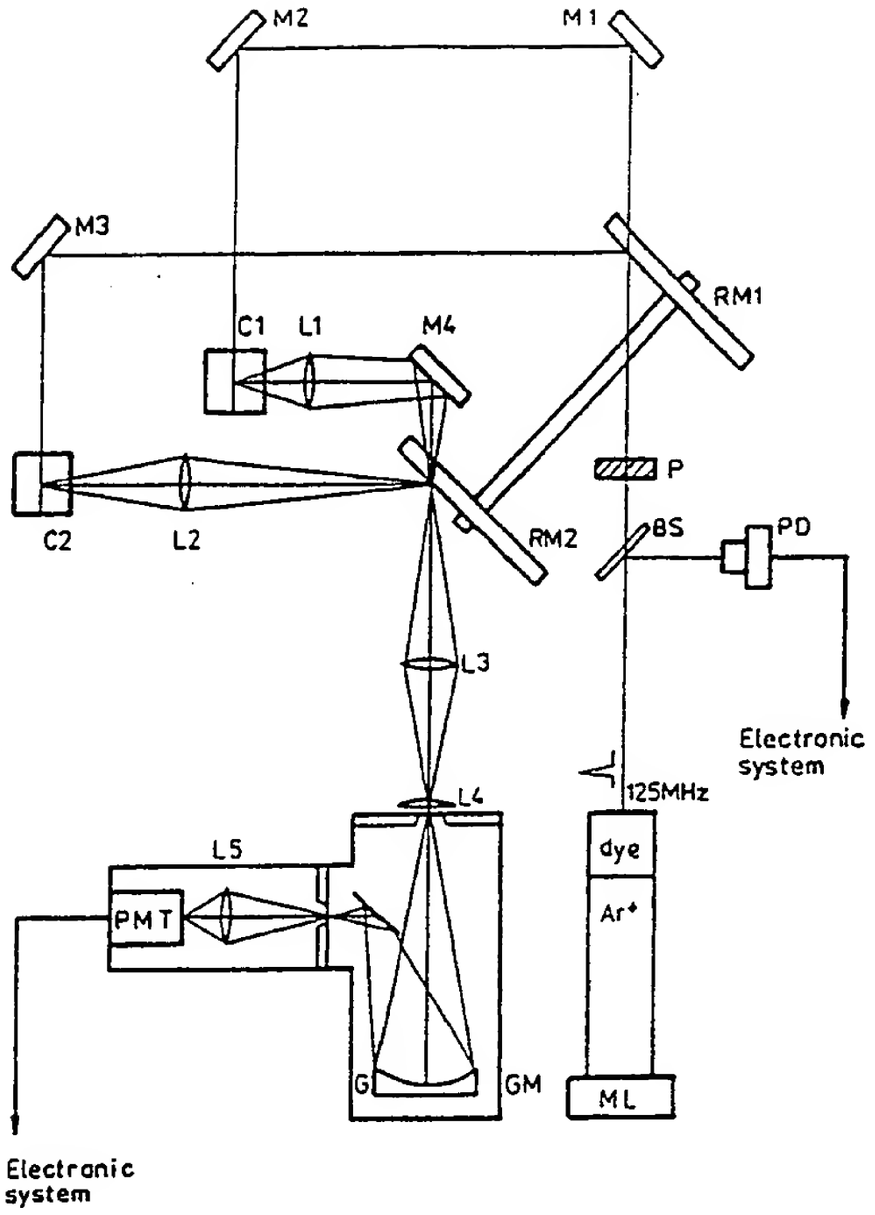

FIG. 2. Optical arrangement (Ar^+ = argon-ion laser, ML = mode locker, M1...M4 = plane mirrors, RM1, RM2 = rotating sector mirrors, C1, C2 = cuvettes, L1...L5 = lenses, P = $\lambda/4$ plate, BS = beam splitter, PD = photodiode, GM = grating monochromator, PMT = photomultiplier tube).

the time stop mode an automatic overflow correction can be used. In this case up to 2^{31} photons/channel can be recorded. Without overflow correction overflowing parts of the result are limited to $2^{15}-1$ photons/channel.

B. SPC oscilloscope

The instrument makes it possible to record decay curves with collection times down to 0.1 s. In the oscilloscope mode the shape of light pulses is measured repeatedly in short intervals and displayed on the screen. Due to the very high count rate this "SPC oscilloscope" allows the inspection of light signals with high time resolution and single-photon sensitivity. The application of the oscilloscope mode to adjust PMT voltage dividers and optical systems is supported by displaying the location of the curve maxima, the FWHM, and the total number of counts.

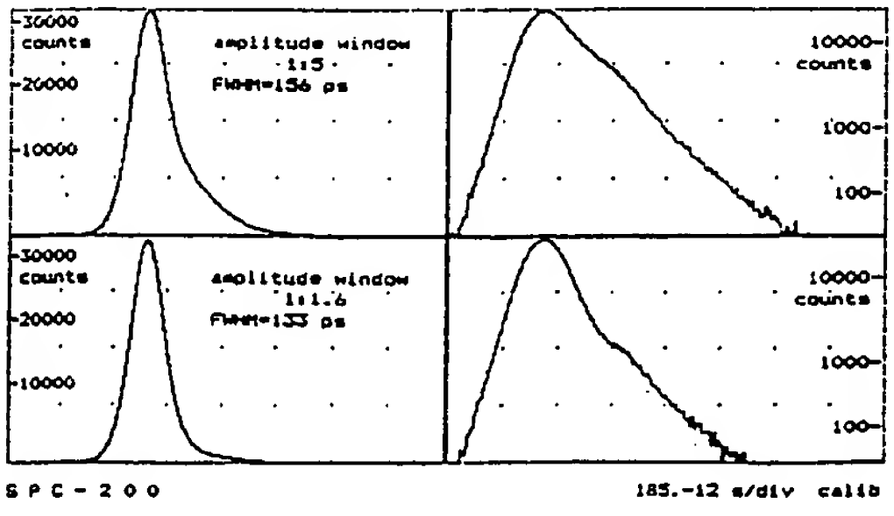

FIG. 3. Response to the dye laser pulses at 585 nm for input amplitude windows of 1:5 and 1:1.6, linear and logarithmic scale; photomultiplier adjusted for smooth impulse response and high count efficiency.

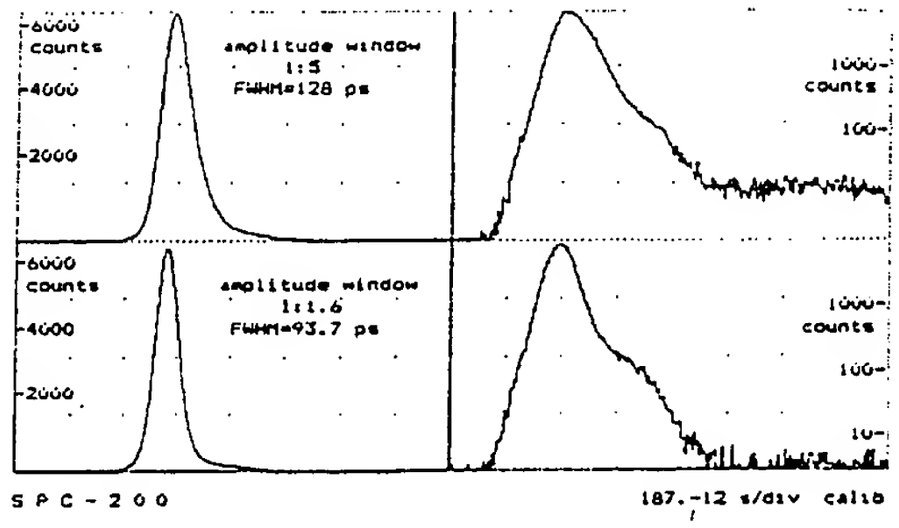

FIG. 4. Response to the dye laser pulses at 585 nm for input amplitude windows of 1:5 and 1:1.6, linear and logarithmic scale, photomultiplier adjusted for minimum FWHM.

C. Multidecay mode

Due to the high count rates and the high capacity of the internal memory (1 MB RAM), sequences of curves depending on external parameters (wavelength, time from the start of the experiment, or another parameter programmed by the user) can be recorded. The result is a three-dimensional display of light intensity versus time and the external parameter.

D. Intensity measurement mode

In this mode, the intensity is averaged in four time windows, and the signal is recorded as a function of the wavelength, the time from the start of the experiment, or another parameter programmed by the user. A possible application of this operation mode is the registration of time-resolved spectra. In this case the dependence of the PMT sensitivity on the wavelength is corrected by the software.

E. Quad channel SPC

A multiplexed measurement of up to four signals is possible in all operating modes. This requires a suitable optical system multiplexing the different optical signals onto the PMT and delivers two channel control signals to the electronic system. The photons are counted into different memory parts depending on the control signals. The use of only one PMT for all signal channels avoids problems with different response functions. Multiplexing technique using different PMTs[2] usually causes matching problems when it is applied to short excitation pulses.

F. Lock-in SPC

The time-correlated single-photon counting can be combined with a digital lock-in technique.[9,10] In this case

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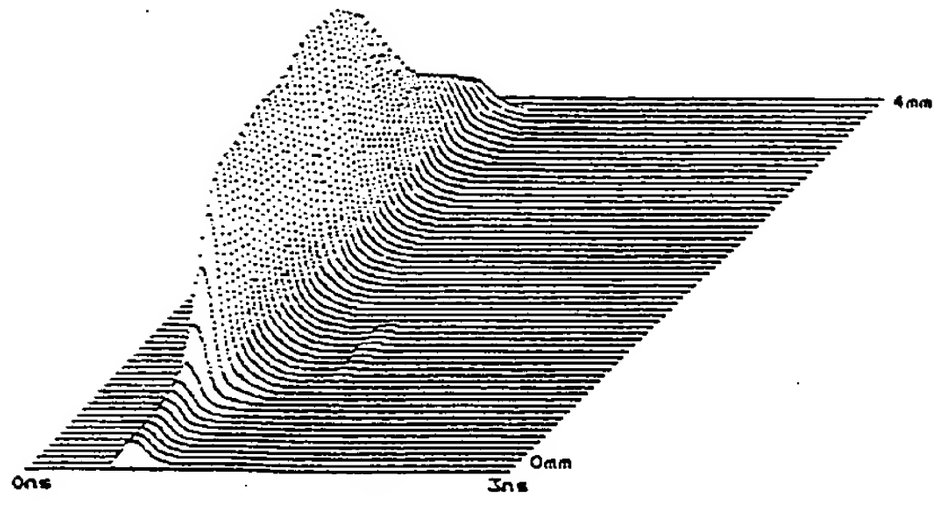

FIG. 5. Impulse response as a function of the position on the photocathode.

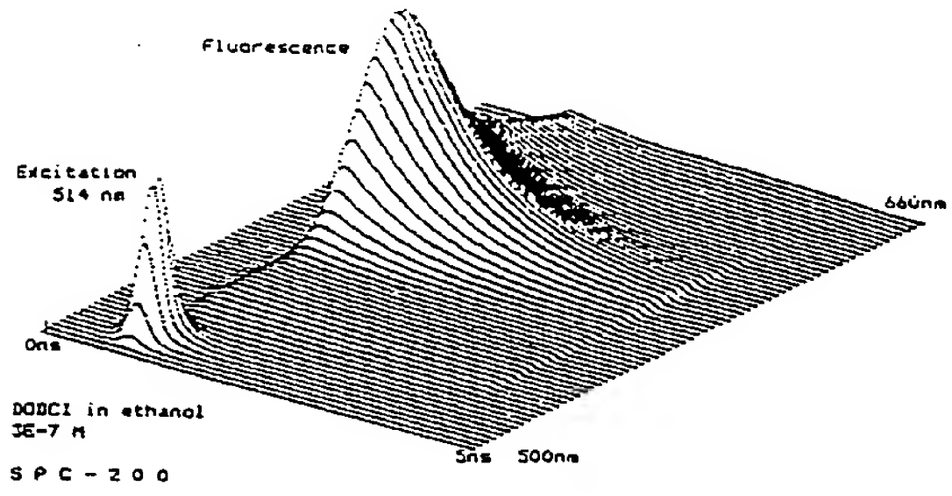

FIG. 7. Fluorescence behavior of 3×10^{-7} M DODCI/ethanol as a function of time and wavelength.

the events are added or subtracted in the memory depending on the lock-in reference signal. The lock-in SPC allows a supression of the PMT background pulses or of stray light if the sample under investigation is excited alternately with a reference object. The lock-in SPC capability can be used in all modes. In the measurement of light pulse shapes, however, there may be different count lasers and different pile-up effects for the sample and of the reference channels, respectively. In such cases considerable errors would affect the result of the direct subtraction procedure. This could be avoided by using the reference signal to store the two measurements in different memory parts, by separately correcting the two sets of accumulated data for the pile-up effect, and finally by performing the subtraction.

G. Data processing and auxiliary software

For data display and processing some useful routines have been implemented:

(1) Curve display: Linear and logarithmic scales, cursor control, numerical display of selected curve points, baseline correction.

(2) Fourier transform: FFT, inverse FFT, amplitude and power spectra, multiplication and division of complex FFT spectra.

(3) Curve manipulation: Smoothing, curve arithmetics, differentiation, integration, interpolation.

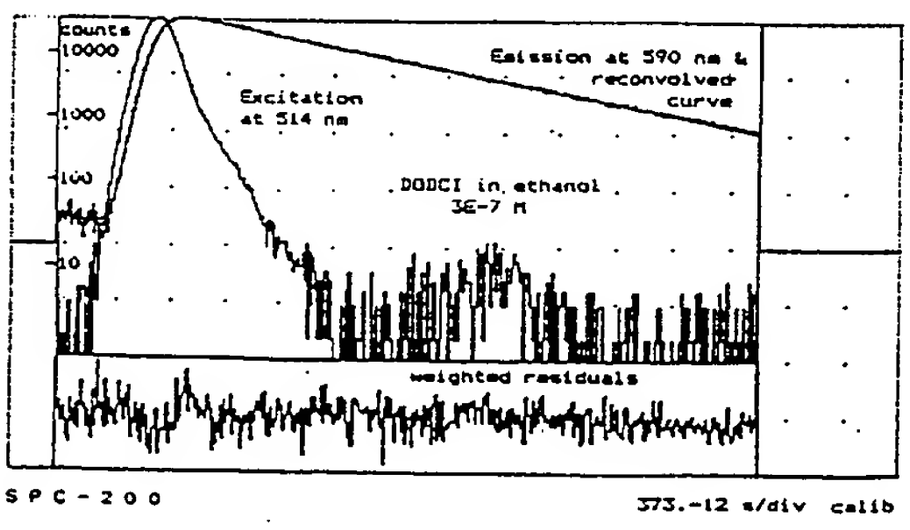

FIG. 6. Fluorescence decay of 3×10^{-7} M DODCI/ethanol, instrumental response, monoexponential fit and weighted residuals.

(4) Deconvolution and depolarization calculation using single exponential decay algorithm.

(5) Display of multidecay results: Manipulation of displayed patterns, smoothing, calculation of sections for processing by curve manipulation.

IV. OPTICAL SYSTEM

The optical arrangement is shown in Fig. 2. An ILA 120 argon-ion laser (Jenoptik GmbH) with an AOM 100 acousto-optical modelocker (Zentrum für wissenschaftlichen Gerätebau, Berlin) and a synchronously pumped rhodamin 6G dye laser are used for sample excitation. The laser operates at 125 MHz repetition rate.

The optical arrangement contains two rotating sector mirrors, RM1 and RM2, on a common axis. RM1 feeds the exciting laser beam via the mirrors M1...M3 alternately to the cuvettes C1 and C2. RM2 and M4 reflect the fluorescence light of C1 or C2 into the grating monochromator GM. The lenses L1 and L2 image the light spots produced in the cuvettes onto the plane of RM2. The lens L3 focuses this image onto the monochromator entrance slit. L4 images L3 onto the grating to achieve uniform illumination. The light from the exit slit is focused onto the photomultiplier cathode by the lens L5.

The $\lambda/4$ plate P converts the linear polarization of the laser beam into a circular polarization. This reduces fluorescence depolarization effects and the influence of back reflections from the optical system to the laser.

The photomultiplier tube is an 18 ELU-FM (Soviet Union). This tube is intended primarily for high-speed pulse applications at output pulse currents up to 2.5 A. The device has a very compact construction and can be operated at a supply voltage as high as 5 kV. Up to 40% of the overall supply voltage has been applied between the cathode and the first dynode. The 18 ELU-FM contains two parallel nine-stage dynode chains. The signal is split at the first dynode and collected at the common anode. The cathode has an active area of 4×45 mm^2 and a sensitivity range from 300 to 850 nm (multialkali cathode). The dark count rate ranges from 50 to 500 s^{-1}. The single electron pulses have a FWHM of 2 ns and an average peak current of 1 mA.

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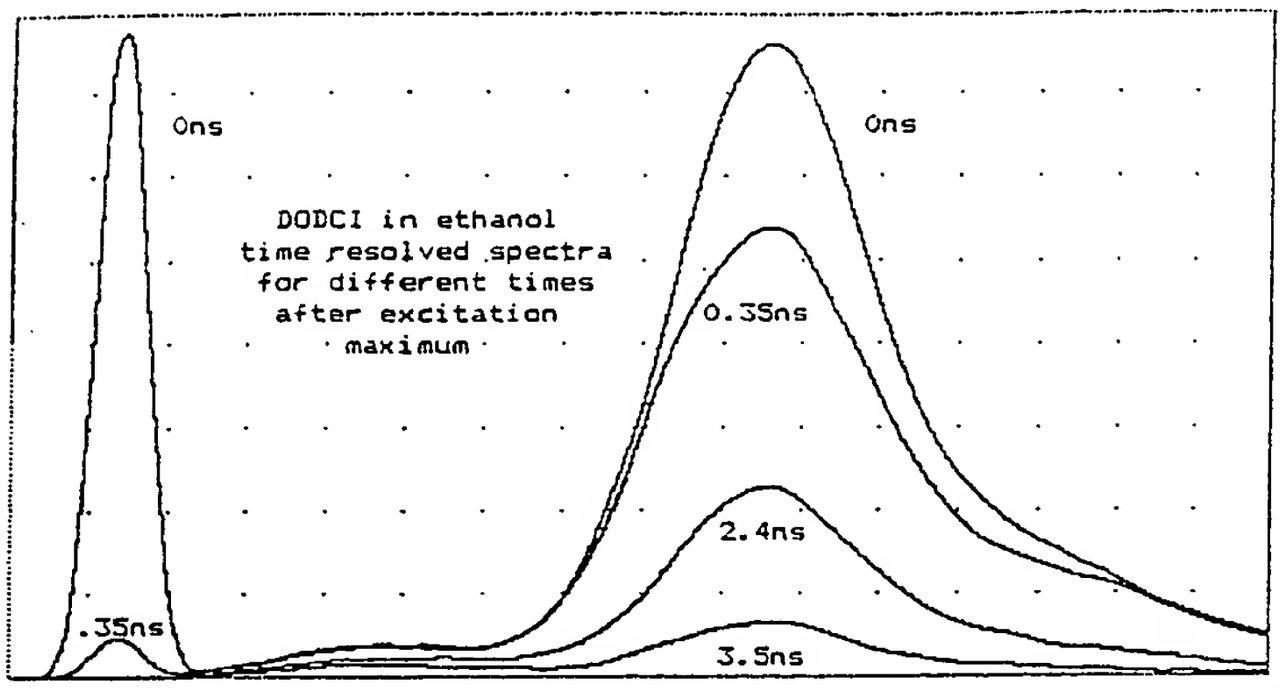

FIG. 8. Time-resolved fluorescence spectra derived from Fig. 6 at different times after excitation maximum.

V. TESTS IN APPLICATIONS

Figures 3 and 4 show the system response to the dye-laser pulse for different lower-level settings of the discriminator DIS1 and for an illuminated cathode area of 4×15 mm^2. In Fig. 3 the voltages at the four focusing electrodes of the PMT have been adjusted to achieve a smooth response and a high count efficiency. For DIS1 amplitude windows of 1:5 and 1:1.6 FWHM values of 156 and 133 ps are achieved. The count rate at the two discriminator settings is 0.8 and 0.1 compared to the true CFT count rate.

In Fig. 4 the focusing voltages have been adjusted for maximum time resolution at the cost of a 50% loss in sensitivity. For amplitude windows of 1:5 and 1:1.6 the FWHM is reduced to 128 and 93.7 ps. The response shows a slow tail due to low amplitude afterpulses. This tail turns into a secondary maximum at wider amplitude windows. Because the shape of the tail strongly depends on the focusing voltages, we suppose that it is due to scattered electrons rather than to propagation delay differences in the two parallel dynode chains.

Figure 5 shows the dependence of the impulse response on the spatial position at the photocathode. A 0.2-mm-diam light spot has been scanned perpendicularly across the 4×45-mm^2 cathode slit. Compared to other results[12,13] the spatial variation of the response shape is very small.

The FWHM of the instrument response is comparable to or better than the best results achieved with conventional photomultipliers in practical applications.[12,13] The dependence of the response on the size of the illuminated cathode area is surprisingly low. This makes it possible to exploit in practice the resolution of the instrument with good reproducibility of the experimental results. The reproducibility is in fact of greater value than the absolute value of the FWHM. Microchannel-plate photomultipliers[14,15] or avalanche photodiodes[7,15] could reduce the FWHM to values below 60 ps, but at the expense of limited count rate or high dark count rate,[6] respectively.

The sample for fluorescence decay time experiments was 3,3'-diethyloxadicarbocyanin-iodid (DODCI, Lambda-Physik) in purified ethanol. To measure the instrumental response function at the excitation wavelength, a special milk solution was used as scatterer. Care was taken to maintain precisely the same optical path geometry for scattered light as for fluorescence.

Figure 6 shows a decay curve of a 3×10^{-7} M DODCI solution measured at 590 nm and the excitation pulse measured at 514 nm with a scattering solution. The fitting to a monoexponential decay function yields a decay time constant of 1.03 ns. The reconvolved curve is in good agreement with the experimental curve and the weighted residuals show a satisfactory quality of the fit.

Figure 7 shows a sequence of decay curves measured with the emission wavelength as a parameter. The collection time was 2 s for each curve, resulting in an overall measuring time of 128 s. From Fig. 7 single decay curves or time-resolved spectra can be obtained. Figure 8 shows time-resolved spectra at different times after the maximum

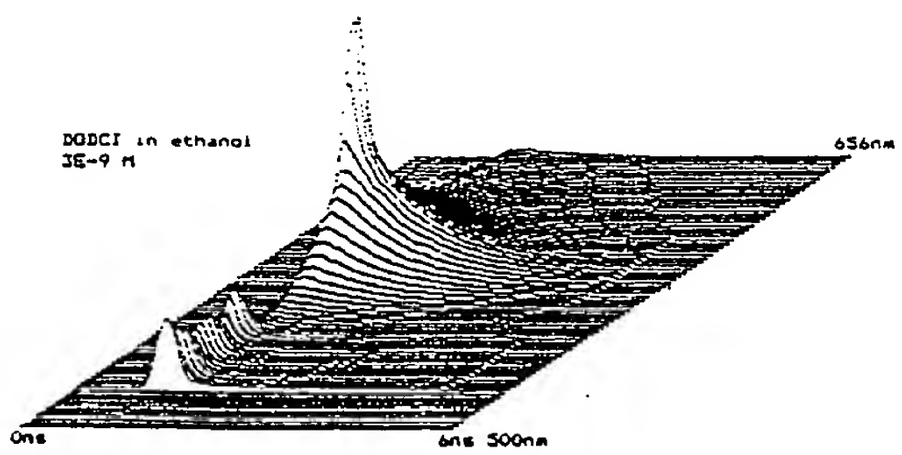

FIG. 9. Fluorescence of DODCI in ethanol, 3×10^{-9} M, with Raman lines of the solvent.

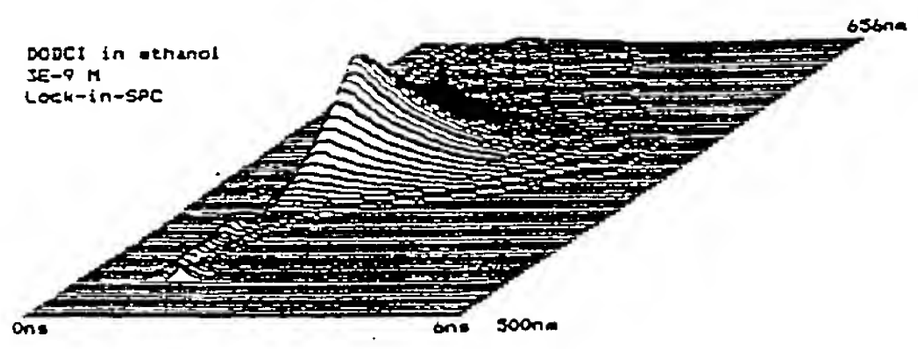

FIG. 10. Fluorescence of DODCI in ethanol, 3×10^{-9} M, recorded by lock-in SPC.

of the excitation pulse. The results of multidecay measurement contain complete information on the fluorescence behavior of the sample. This considerably improves the reliability of experimental results.

Figures 9 and 10 show an application of the lock-in capability. In these measurements the concentration was reduced to 3×10^{-9} M. At this concentration the Raman spectrum of the solvent appears in the result, interfering with the fluorescence data (Fig. 9). In Fig. 10 the digital lock-in technique has been applied. The sample has been measured in alternation with a solvent cuvette as reference. As can be seen, the Raman lines are considerably reduced. A weak fluorescence signal of a solvent contamination at the short-wavelength side of the DODCI fluorescence is also eliminated. The Raman signal is suppressed by a factor of about 10. This value is due to the time and intensity mismatching of the two optical channels in the optical system. The suppression of the unwanted signal in the electronic system itself is much better, so that the matching of the optical channels seems to be the main problem associated with lock-in SPC measurements.

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